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From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 05 April 2000 (05.04.00)	
International application No. PCT/US99/13858	**Applicant's or agent's file reference** 9426-004-228
International filing date (day/month/year) 18 June 1999 (18.06.99)	**Priority date** (day/month/year) 19 June 1998 (19.06.98)
Applicant HERR, John, C. et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

19 January 2000 (19.01.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer S. Mafla
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PATENT COOPERATION TREATY

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INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

REC'D 14 AUG 2000
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Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
9426-004-228		
International application No. PCT/US99/13858	International filing date *(day/month/year)* 18 JUNE 1999	Priority date *(day/month/year)* 19 JUNE 1998
International Patent Classification (IPC) or national classification and IPC Please See Supplemental Sheet.		
Applicant UNIVERSITY OF VIRGINIA PATENT FOUNDATION		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 4 sheets.

   ☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority. (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 0 sheets.

3. This report contains indications relating to the following items:

   I ☒ Basis of the report

   II ☐ Priority

   III ☐ Non-establishment of report with regard to novelty, inventive step or industrial applicability

   IV ☐ Lack of unity of invention

   V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

   VI ☐ Certain documents cited

   VII ☐ Certain defects in the international application

   VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
19 JANUARY 2000	11 JULY 2000
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer PATRICK NOLAN Telephone No. (703) 308-0196

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US99/13858

I. Basis of the report

1. With regard to the **elements** of the international application:*

[X] the international application as originally filed

[X] the description:
pages 1-78, as originally filed
pages NONE, filed with the demand
pages NONE, filed with the letter of ____

[X] the claims:
pages 79-81, as originally filed
pages NONE, as amended (together with any statement) under Article 19
pages NONE, filed with the demand
pages NONE, filed with the letter of ____

[X] the drawings:
pages 1-20, as originally filed
pages NONE, filed with the demand
pages NONE, filed with the letter of ____

[X] the sequence listing part of the description:
pages NONE, as originally filed
pages NONE, filed with the demand
pages NONE, filed with the letter of ____

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language ____ which is:

[ ] the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).

[ ] the language of publication of the international application (under Rule 48.3(b)).

[ ] the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

[ ] contained in the international application in printed form.

[ ] filed together with the international application in computer readable form.

[ ] furnished subsequently to this Authority in written form.

[ ] furnished subsequently to this Authority in computer readable form.

[ ] The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[ ] The statement that the information recorded in computer readable form is identical to the writen sequence listing has been furnished.

4. [X] The amendments have resulted in the cancellation of:

[X] the description, pages N/A

[X] the claims, Nos. N/A

[X] the drawings, sheets/~~fig~~ N/A

5. [X] This report has been drawn as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).**

** Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).*

***Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.*

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US99/13858

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. statement

Novelty (N)	Claims	1-6, 10-11, 13 and 15-18	YES
	Claims	7-9, 12 and 14	NO
Inventive Step (IS)	Claims	1-6, 13, and 15-18	YES
	Claims	7-12, and 14	NO
Industrial Applicability (IA)	Claims	1-18	YES
	Claims	NONE	NO

2. citations and explanations (Rule 70.7)

Claims 7-9, 12 and 14 lack novelty under PCT Article 33(2) as being anticipated by U.S. Patent No. 5,641,487.

The '487 patent teaches making antibody to the ZP3 protein, an egg surface protein, and teaches a method to screen the ability of the antibody to bind the ZP3 protein, (see columns 27-29, in particular).

Claims 10-11 lack an inventive step under PCT Article 33(3) as being obvious over U.S. Patent No. 5,641,487.

The '487 patent has been discussed supra. The claimed invention differs from the prior art teachings by the recitation of making a monoclonal antibody to an egg surface protein. However, it would have been prima facie obvious to one of skill in the art at the time the invention was made to be motivated to make a monoclonal antibody to egg surface protein since it is art recognized that monoclonal antibodies dramatically increase the specificity of antibodies to the protein.

Claims 1-6, 13 and 15-18 meet the criteria set out in PCT Article 33(2)-(4), because the prior art does not teach or fairly suggest the antigenic fusion protein or methods of its use.

Claims 1-18 meet the criteria set out under PCT Article 33(4).

---------- NEW CITATIONS ----------

NONE

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

CLASSIFICATION:
The International Patent Classification (IPC) and/or the National classification are as listed below:
IPC(6): A61K 39/00; C07K 1/00; C12Q 1/00, 1/68; G01N 33/53 and US Cl.: 424/184.1; 435/4, 6; 436/547; 530/395, 403

I. BASIS OF REPORT:

5. (Some) amendments are considered to go beyond the disclosure as filed:
NONE

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/13858

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 39/00; C07K 1/00; C12Q 1/00, 1/68; G01N 33/53

US CL : 424/184.1; 435/4, 6; 436/547; 530/395, 403

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/184.1; 435/4, 6; 436/547; 530/395, 403

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

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C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,641,487 A (DEAN ET AL) 24 JUNE 1997(24/6/97), see enrie document.	1-18

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
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"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 12 AUGUST 1999	Date of mailing of the international search report 07 OCT 1999
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Form PCT/ISA/210 (second sheet)(July 1992)*

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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Published
With international search report.

(54) Title: EGG SURFACE PROTEINS AND METHODS FOR USE FOR MODULATING FERTILITY

(57) Abstract

The present invention is directed to egg surface antigens useful for producing antibodies which bind epitopes on the egg surface and modulate fertility. The invention encompasses compositions and methods for immunizing an individual for production of antibodies against egg surface antigens. The invention is based on the discovery of egg surface antigens involved in egg–sperm binding and fusion. Methods are provided for the use of such antigens in methods for sterilization of female animals. Methods are further provided for the use of egg surface antigens to generate antibodies useful for temporary, reversible contraception methods. Methods are further provided for the use of anti–idiotypic monoclonal antibodies which mimic egg surface epitopes to actively immunize a mammal against pregnancy.

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EGG SURFACE PROTEINS AND METHODS FOR USE FOR MODULATING FERTILITY

This application claims priority under 35 U.S.C. §119(e) to provisional patent application no. 60/089,950, filed June 19, 1998, the entire contents of which is incorporated herein by reference in its entirety. This invention was made with government support under grant numbers U54-HD29099, P30-28934, P32-DK07642, T32-HD07382, and F32-HD08002 awarded by the National Institutes of Health. The government has certain rights in the invention.

1. INTRODUCTION

The present invention is directed to egg surface antigens useful for producing antibodies which bind epitopes on the egg surface and modulate fertility. The invention encompasses compositions and methods for immunizing an individual for production of antibodies against egg surface antigens. The invention is based on the discovery of egg surface antigens involved in egg-sperm binding and fusion. Methods are provided for the use of such antigens in methods for sterilization of female animals. Methods are further provided for the use of egg surface antigens to generate antibodies useful for temporary, reversible contraception methods. Methods are further provided for the use of anti-idiotypic monoclonal antibodies which mimic egg surface epitopes to actively immunize a mammal against pregnancy.

2. BACKGROUND OF THE INVENTION

There is increasing interest in developing an immunological approach to contraception for humans and sterilization for animal populations. A contraceptive vaccine would provide many advantages over currently available methods of contraception. Methods of contraception such as hormone therapies and chemical or mechanical barriers against fertilization have serious drawbacks, such as undesirable side effects and less than complete effectiveness. For example, side effects of hormonal therapies such as the pill include cancer, and in the case of mechanical barriers, increased susceptibility to infection. In addition, contraceptive vaccines would further be useful for fertility control of animal populations, where long-term or permanent sterilization, without the need for frequent intervention, is desirable. For example, such long-term sterilization would be useful for

controlling fertility in human beings or agriculturally important livestock, such as cattle and pigs. Further, contraceptive vaccines would be useful for permanent sterilization regimes useful for pest control, such as for sterilization of rodents or other unwanted populations.

While there has been much interest in the development of immunocontraceptives, the focus has been, until recently, on the development of immunocontraceptives directed against sperm surface antigens, or on already known peptide hormones such as human chorionic gonadotropin and follicle stimulating hormone. One obstacle to the development of an effective egg surface antigen based immunocontraceptive vaccine has been the lack of knowledge regarding the molecular identities of egg surface proteins known to be directly involved in the fertilization process.

Mammalian fertilization may be defined as a series of gametic interactions in which capacitated sperm must first penetrate the cumulus cells and zona pellucida (the egg vestments), then bind to and fuse with the egg plasma membrane (oolemma). The initial binding event between gametes is known as primary binding and occurs, in the mouse model, when the zona pellucida protein, ZP3, binds to a receptor(s) on the sperm (reviewed in (McLeskey et al., 1998, Int. Rev. of Cytol. 177: 57-113). This binding event also initiates the acrosome reaction in which hydrolytic enzymes are released from the acrosomal compartment and act on the zona pellucida to facilitate penetration of the zona pellucida by sperm. Zona penetration is known as secondary binding and is mediated by the zona protein, ZP2, and one or more molecules on the inner acrosomal membrane (reviewed in Snell and White, 1996, Cell 85: 629-637).

Upon emergence from the zona pellucida, sperm then cross the perivitelline space and bind to and fuse with the oolemma. The molecular basis of sperm-oolemma binding and fusion has yet to be fully elucidated; however, recent evidence has demonstrated that integrins are involved in the interaction. Almeida et al. (1995, Cell 81: 1095-1104) found that when oocytes were treated with monoclonal antibodies against the egg surface integrin $\alpha 6\beta 1$, mouse sperm-oolemma binding was reduced. Further, these investigators reported that somatic cells which express $\alpha 6\beta 1$ bind mouse sperm avidly while somatic cells that lack $\alpha 6$ or $\beta 1$ do not. A proposed sperm surface ligand for $\alpha 6\beta 1$ is fertilin. Fertilin contains a domain homologous to a family of integrin ligands known as disintegrins (Blobel et al., 1992, Nature 356: 248-252), which suggest a cell adhesion function for the molecule. Also, recombinant fertilin is known to bind to the oolemma (Evans et al., 1997, Dev. Biol. 187:79-93), with both monoclonal antibodies to fertilin (Primakoff et al., 1987, J. Cell. Biol. 104: 141-149) and fertilin peptide analogs (Almeida et al., 1995, Cell 81: 1095-1104; Evans et al., 1995, J. Cell. Sci. 108: 3267-3278) blocking sperm-oolemma binding and fusion.

Sperm-egg binding and fusion is likely to require multiple receptor-ligand interactions and other oolemmal proteins are likely to be involved in the fertilization process. In fact, there is indirect evidence implicating other oolemmal proteins in sperm-egg interaction. A purified sperm-associated protein (protein DE) which is involved in fusion in the rat, binds to the surface of zona-free rat oocytes (Cohen et al., 1996, Biol. Reprod. 55: 200-206). Another putative oolemmal sperm receptor is removed from the surface of radioiodinated mouse eggs following trypsin treatment and reappears on the egg surface after 3-6 h of culture (Kellom et al., 1992, Mol. Reprod. Dev. 33: 46-52). The reappearance of this 94 kDa protein on the egg surface coincides with the ability of the trypsin-treated eggs to be penetrated by sperm.

Glycosyl-phosphatidylinositol (GPI)-anchored proteins may play a key role in gamete interaction. GPI-anchored proteins possess a covalently linked glycosylated phosphatidylinositol moiety which serves to attach the protein portion of the molecule to the cell surface lipid bilayer (Low and Saltiel, 1988, Science, 239: 268-275). Proteins linked to the cell surface via a phosphatidylinositol anchor are known to be involved in a wide variety of cellular functions including T cell activation, hydrolysis of extracellular matrix proteins, transduction of extracellular stimuli, and cell-cell adhesion (reviewed in Low and Saltiel, 1988, Science, 239: 268-275). GPI-anchored proteins can be released from the cell surface by treatment of cells with the highly specific enzyme phosphatidylinositol-specific phospholipase C (PI-PLC) (Low and Finean, 1978, Bioch. Biophys. Acta. 508: 565-570). Therefore, treatment of intact cells with PI-PLC has become a useful tool to characterize the released proteins and to investigate the role of GPI-anchored proteins in cell function.

Mouse sperm surface hyaluronidase (also known as PH-20) is a GPI-anchored protein and is thought to aid sperm in passage through the cumulus oopherous and possibly the zona pellucida by hydrolyzing the extracellular matrix protein, hyaluronic acid (Gmachl and Kreil, 1993, Proc. Nat. Acad. Sci. USA 90: 3569-3573); (Myles and Primakoff, 1997, Biol. Reprod., 56: 320-327). Sperm agglutination antigen-1 (SAGA-1) is another sperm surface protein which has been shown to be a GPI-linked. While its role in fertilization has yet to be elucidated, in vitro assays have demonstrated that anti-SAGA-1 monoclonal antibodies agglutinate human sperm (Diekman et al., 1997, Biol. Reprod. 57: 1136-1144).

Other reports suggest the presence of GPI-anchored proteins on oocytes. A PI-PLC sensitive GPI-linked protein, N-acetylglucosaminidase, is cleaved from the surface of Ascidians eggs following fertilization, and occupies sperm binding sites on the vitelline coat to protect the egg against polyspermy (Lambert and Goode, 1992, Dev. Biol. 154: 95-

100). Less is known about the existence of GPI-anchored proteins on the mammalian egg surface. Hirao and Yanagimachi, (1978, Gam. Res. 1: 3-12) treated hamster oocytes with a variety of enzymes including; proteases, lipases, and glycosidases and found that only phospholipase C (PLC) blocked sperm-egg fusion. Boldt et al. (1988, Biol. Reprod. 39: 19-27) treated mouse oocytes with PLC and found that the enzyme inhibited sperm-egg binding but did not inhibit sperm-egg fusion. However, due to the broad specificity of PLC for a variety of phospholipids, it has not been possible to determine if the inhibitory effects on fertilization were due to the specific release of oolemmal GPI-anchored proteins. In the only previous report which investigated the effects of PI-PLC on mammalian fertilization, Clark and Koehler (1988, Gam. Res. 19: 339-348) treated hamster oocytes with PI-PLC for 3 min and found that the enzyme had a slight, but significant, inhibitory effect on sperm-egg fusion.

The hamster oocyte is unique in that zona-free eggs from other species such as the mouse, rat, and guinea pig do not incorporate heterologous sperm as readily (Yanagimachi, 1972, J. Reprod. Fertil., 28: 477-480; Hanada and Chang, 1976, J. Reprod. Fertil., 46: 239-241; and Quinn, 1979, 210: 497-506). Because of this promiscuity, the zona-free hamster egg has been used extensively in the sperm penetration assay (SPA) to assess the fertilizing capacity of human spermatozoa (Yanagimachi et al., 1976, Biol. Reprod., 15: 471-476; Rodgers et al., 1979; Liu and Baker, 1992, Fertil. Steril., 59: 698-699). In spite of the widespread use of this assay, the molecular interactions which occur between the human sperm and hamster oocyte during gamete interaction remain largely unknown. Presumably, however, there are molecules on the hamster egg plasma membrane (oolemma) which specifically interact with molecules on the human sperm plasma membrane during sperm-egg binding and fusion.

There have been a number of recent attempts to produce contraceptive vaccines directed against egg antigens (U.S. Patent Nos. 5,820,863, 5,641,487, 5,637,300, and 4,996,297). To date, because of their relative abundance and accessibility to immunodetection, the focus has been on identifying zona pellucida epitopes. However, results from fertility trials in several species have shown that ovarian histopathology is often observed in ZP3 immunized animals. Therefore, commercial contraceptive companies have lost interest in zona proteins as contraceptive immunogens.

A new approach for an effective contraceptive vaccine that specifically targets antigens directly involved in the fertilization process is needed. However, to date, there are no reports of vaccines directed against egg protein(s) directly involved in the process of sperm-egg fusion step which is required for fertilization.

Citation or discussion of a reference herein shall not be construed as an admission that such is prior art to the present invention.

3. SUMMARY OF THE INVENTION

The present invention is directed to egg plasma membrane antigens useful for producing antibodies which will bind epitopes on the egg surface and modulate fertilization. The invention is based on the discovery that egg surface GPI-anchored proteins are directly involved in egg-sperm fusion, a key step in fertilization.

The invention further encompasses compositions and methods for immunizing an individual for production of antibodies against egg surface antigen(s). The invention is based on the discovery of a conserved set of egg surface antigens, a subset of which are released from the follicular membrane upon treatment of the membrane with PI-PLC. Methods are also provided for the use of antibodies against such antigens for active immunization, or sterilization, of female animals, by induction of a T-cell attack on the egg. These methods are particularly useful in cases where permanent sterilization is desired, for example, for sterilization of animal populations. Methods are further provided for the use of egg surface antigens to generate antibodies useful for passive immunization that can be used for reversible contraception methods. Methods are further provided for the use of anti-idiotypic monoclonal antibodies which mimic egg surface epitopes to actively immunize a mammal against pregnancy.

In another embodiment, the invention comprises a monoclonal antibody against an egg surface protein, novel hybridoma cells which express such antibodies; and methods for immunocontraception utilizing such monoclonal antibodies.

The invention further encompasses pharmaceutical compositions comprising the antigen preparation of the invention and immune-response enhancing components, together with pharmacologically acceptable carrier.

4. BRIEF DESCRIPTION OF THE FIGURES

Figure 1 (A-E) PI-PLC treatment of gametes has no effect on sperm-zona pellucida binding, however, fertilization of zona-intact oocytes is blocked. Both sperm and eggs were separately treated with 1 U per ml PI-PLC for 30 min followed by gamete co-incubation in the presence of PI-PLC. To evaluate the effects of PI-PLC on sperm-zona binding, the oocytes were washed following 1 h gamete co-incubation and prepared for observation (see methods in Example in Section 6). A single focal plane for each oocyte was selected in

which the widest diameter of the zona pellucida could be visualized and the number of sperm in that focal plane was determined. No difference in sperm-zona pellucida binding was noted between the control group treated with inactivated PI-PLC (A) and the PI-PLC treated group (B). To evaluate the effects of PI-PLC on fertilization, the oocytes were washed following 2 h gamete co-incubation and incubated overnight. Following 24 h incubation, a significant decrease in the number of fertilized oocytes (as determined by cleavage) was noted in the treatment group (D) compared to the control group (C). In Figs. 1(C) and 1(D), zygotes were treated with 1μm Hoechst # 33342 for 10 min and washed to visualize sperm which accumulated in the perivitelline space of the PI-PLC treated group (Fig. 1D, inset). Images for Figs. 1(A) and (B) (200X) were recorded using phase contrast. Images for Figs. 1(C) and (D) (200X, insets 400X) were recorded using combined phase contrast and fluorescent microscopy. Results are shown quantitatively in the histogram (E). Bars represent means +/- the standard deviation of three individual experiments. N = total number of oocytes per group. * = $p \leq 0.05$ (students T test).

Figure 2 (A-E) Treatment of sperm with PI-PLC prior to incubation with zona-free oocytes has no effect on sperm-egg binding and fusion whereas treatment of eggs with PI-PLC inhibits sperm-egg binding and fusion. Zona-free oocytes were pre-loaded with 1 μm Hoechst #33342 for 10 min and washed. Next, either sperm (A,B) or zona-free eggs (C,D) were treated with 1 U per ml PI-PLC for 30 min, washed, and the gametes were co-incubated for 40 min. The oocytes were then gently washed and prepared for observation (see methods in the Example given in Section 6). Sperm-egg binding was scored by counting the number of bound sperm per egg using phase contrast. Fusion was scored by counting the number of penetrated sperm heads using combined phase contrast and fluorescent imaging. When only sperm were treated with PI-PLC, no difference was noted in the number of sperm binding to and fusing with the egg when comparing the group treated with inactivated PI-PLC (A) to the PI-PLC treated group (B). However, when eggs were treated with PI-PLC a significant decrease in sperm-egg binding and fusion was observed when comparing the inactivated PI-PLC group (C) with the PI-PLC treated group (D). Several of the PI-PLC treated eggs had numerous bound sperm (see oocyte indicated by arrow on D) yet no fusion was recorded in most of these oocytes. Note: the fluorescent spot seen in each oocyte in (D) is the metaphase plate. Images were recorded at 200X. Results are shown quantitatively in the histogram in Fig 1(E). Bars represent means +/- the standard deviation of three individual experiments. N = total number of oocytes per group. * = $p \leq 0.05$ (students T test).

Figure 3 Treatment of oocytes with PI-PLC inhibits sperm-egg binding and fusion in a dose-dependent manner. Zona-free eggs were treated with either 5U per ml of heat inactivated PI-PLC, no PI-PLC, or increasing concentrations of PI-PLC for 30 min, washed and co-incubated with untreated sperm for 40 min. The oocytes were gently washed and prepared for observation (see methods in the Example in Section 6). Sperm-egg binding or fusion rates were not inhibited when oocytes were treated with 5U of heat inactivated PI-PLC for 30 min, however when oocytes were treated with increasing concentrations of PI-PLC, binding and fusion rates were inhibited in a dose-dependent manner, with the maximal inhibitory effect on sperm-egg binding observed at 5U/ml and fusion at 1U/ml. Each value represents the mean +/- standard deviation of three individual experiments. N = total number of oocytes per group.

Figure 4 Two-dimensional gel electrophoresis and silver staining reveals that the PI-PLC enzyme preparation is highly purified. One μg of the enzyme preparation was separated by two-dimensional electrophoresis and the gel was silver stained. Results show one prominent protein spot (~MW 30 kDa, ~pI 6) and several smaller protein spots immediately surrounding the prominent protein. See the Example in Section 6 for complete experimental details.

Figure 5 (A-B) Mouse eggs treated with PI-PLC can be artificially activated with calcium ionophore A23187. Zona-free eggs were pre-loaded with Hoechst #33342, washed, and treated with PI-PLC for 30 min. A small number of oocytes from the control group (A, inset) and treatment group (B, inset) were observed to ensure that oocytes had remained in metaphase II arrest following treatment. Oocytes were then activated with 0.5 μm calcium ionophore A23187 for 5 min, washed and cultured for 40 min. There was no observable difference in the number of eggs which resumed meiotic cell division (as determined by oocytes progressing from metaphase II arrest to anaphase II or telophase) in the group treated with inactivated PI-PLC (A) compared with the PI-PLC treated group (B). This experiment was repeated three times. Representative images were recorded using combined dual phase contrast and fluorescent microscopy (200X; inset, 100X).

Figure 6 (A-C) Treatment of zona-free oocytes with PI-PLC does not affect recognition of the α6β1 integrin by its cognate antibody. Fluorescent beads (1μM) were coated with α6β1 antibodies then incubated with zona-free eggs that were either untreated or treated with 1 U per ml PI-PLC. No difference was observed in the number of anti-α6β1 coated beads bound per egg when comparing the control group (A) with the PI-PLC treated group (B). Few

beads bound when beads were coated with an equivalent concentration of control antibody and incubated with untreated eggs (C).

Figure 7 (A-D) Two-dimensional avidin blots of biotinylated egg surface proteins demonstrate that treatment of zona-free oocytes with PI-PLC releases 70 kDa (pI 5) and 35-45 kDa (pI 5.5) protein clusters from the egg surface. (A) The repertoire of sulfo-NHS biotin labeled surface proteins extracted from eggs which were not treated with PI-PLC. The 70 kDa (pI 5) and 35-45 kDa (pI 5.5) protein clusters (indicated by arrowheads) are PI-PLC sensitive. The three protein spots denoted by asterisks bind streptavidin-HRP non-specifically. (B) No biotinylated protein spots were evident in supernatant obtained from oocytes incubated for 30 min in the absence of PI-PLC. (C) The repertoire of biotin labeled surface proteins extracted from PI-PLC treated eggs. Arrows represent the locations of 70 kDa (pI 5) and 35-45 kDa (pI 5.5) protein clusters which decline following PI-PLC treatment. (D) Supernatant from PI-PLC treated eggs reveals proteins (indicated by arrowheads) of similar molecular weights and isoelectric points to those released from the eggs surface following PI-PLC treatment. Two small protein spots at ~ 75-78 kDA and pI 5.5 (arrowheads, in D) were also released from the egg surface into the supernatant following PI-PLC treatment. These proteins were only seen in two of five replications of this experiment.

Figure 8 (A-B) Sperm-egg binding is significantly enhanced and sperm-egg fusion is not effected when human sperm are treated with PI-PLC and incubated with zona-free hamster oocytes. Human sperm were treated with either 1 U/ml PI-PLC or 1 U/ml heat inactivated PI-PLC (95°C, 5 mm) for 30 min, washed, and incubated with untreated zona-free hamster oocytes for 3 h. The oocytes were then gently pipetted to remove loosely bound sperm and the eggs were briefly incubated in acridine orange to stain chromatin. The number of sperm bound per oocyte (A) was then scored using phase contrast microscopy and sperm-egg fusion (B) was scored by counting the number of swollen sperm heads within each oocyte using fluorescent microscopy. Bars represent means +/- the standard deviation of three individual experiments. N = total number of oocytes per group * = $p < 0.05$ (students T test).

Figure 9 (A-B) Sperm-egg binding and sperm-egg fusion is blocked when zona-free hamster oocytes are treated with PI-PLC and incubated with human sperm. Zona-free hamster oocytes were treated with either 1 U/ml PI-PLC or 1 U/ml heat inactivated PI-PLC (95°C, 5 min.) for 30 min, washed and incubated with untreated human sperm for 3h. The

oocytes were then gently pipetted to remove loosely bound sperm and the eggs were briefly incubated in acridine orange to stain chromatin. The number of sperm bound per oocyte (A) was then scored using phase contrast microscopy and sperm-egg fusion (B) was scored by counting the number of swollen sperm heads within each oocyte using fluorescent microscopy. Bars represent means +/- the standard deviation of three individual experiments. N = total number of oocytes per group. *=$p \leq 0.05$ (students T test).

Figure 10 (A-B) Zona-free hamster oocytes can be artificially activated following PI-PLC treatment. To ensure that the PI-PLC preparation was not affecting oocyte viability, zona-free eggs were preloaded with Hoechst #33342, washed, and treated with either 1 U/ml PI-PLC or 1 U/ml heat inactivated PI-PLC (95°C, 5 min) for 30 min. Gocytes were then activated with 0.5 µm calcium ionophore A23 187 for 5 min, washed incubated at 37°C and 5% CO_2 for 3h. The eggs were considered activated if they had advanced from metaphase II arrest to anaphase II or telophase II (with second polar body). There was no observable difference in the number of eggs which resumed meiotic cell division in the group treated with inactivated PI-PLC (A) compared with the PI-PLC treated group (B). Arrows indicate extruded chromatin (stained blue) within the second polar body of oocytes from both groups. This experiment was repeated three times. Representative images were recorded using combined dual phase contrast and fluorescent microscopy (200X). Bar represents 20 µm.

Figure 11 (A-D) Treatment of zona-free hamster oocytes with PI-PLC releases a 25-40 kDa (pI 5-6) protein cluster from the oolemma. Zona-free hamster oocytes were biotinylated, separated into two groups (130 oocytes per group), and either mock treated or treated with 1 U/ml PI-PLC. The supernatants were collected, the eggs were washed, and the egg proteins were extracted in Celis buffer. The egg protein extracts and the proteins from the supernatants were separated by 2-D electrophoresis and electroblotted to nitrocellulose membranes. The membranes were stained with Protogold to visualize the egg proteins (red staining). The membranes were then blocked with 5% milk, probed with strepavidin-HRP, and egg surface proteins were visualized using TMB membrane peroxidase substrate (blue staining). (A) The repertoire of surface labeled oolemmal proteins from mock treated eggs. Small arrowheads indicate surface labeled proteins. Small arrowheads labeled d indicate proteins dually labeled by Protogold and TMB. Three protein trains (t1, t2, and t3) and two protein clusters (c1 and c2) were also surface labeled. The two protein spots denoted by asterisks bound strepavidin-HRP non-specifically. (B) Supernatant from mock treated oocytes. (C) The repertoire of surface labeled oolemmal proteins

following PI-PLC treatment. Arrows labeled c2 represent the location of the 25-40 kDa (pI 5-6) protein cluster which was prominent in extracts of mock treated oocytes. (D) Supernatant from PI-PLC treated oocytes. Arrows indicate a 25-40 kDa (pI 5-6) protein cluster which has a mass and isoelectric point similar to that which is released from the oocyte following PI-PLC treatment. Asterisk indicates PI-PLC isoforms.

Figure 12 (A-B) (A). Nucleotide sequence for the ZP3 human mRNA (SEQ ID NO:1). (B). ZP3 amino acid sequence (SEQ ID NO:2) as derived from the nucleotide sequence of the ZP3 human mRNA.

Figure 13 (A-B) Oolemmal ZP3 (A) partitions in the detergent phase of Triton-S 114 extracts whereas zona matrix ZP3 (B) partitions in the aqueous phase. D, detergent phase. A, aqueous phase.

Figure 14 Localization of ZP3 to the oolemma of zona-free mouse oocytes. On the left a control rat IgG antibody was used; on the right anti-ZP3 (IE-10) was used.

Figure 15 Kidney cells transfected with full length recombinant mouse ZP3 express ZP3 at the cell surface. Stained cells are shown at 200X magnification using phase contrast microscopy, and 200X and 630X using fluorescence microscopy.

Figure 16 (A-B) Specificity of the IE-10 mAb for oolemmal ZP3. Shown are without (A) or with (B) J peptide using contrast and fluorescent microscopy.

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to identification of egg surface proteins, that can be used as immunogens in a vaccine preparation to aid in the modulation of fertility. Described in detail below are the egg surface antigens, methods for their production, and methods for their use to modulate fertility.

5.1 EGG SURFACE PROTEINS AND POLYPEPTIDES

5.1.1 Egg Surface Protein Antigens

The invention relates to polypeptide antigens present on the egg plasma membrane. Such antigens have been identified as molecules directly involved in egg-sperm fusion required for fertilization. These specific glycosyl-phosphatidylinositol (GPI)-

anchored proteins are released from the egg plasma membrane by treatment of eggs with phosphatidylinositol-specific phospholipase C (PI-PLC). Release of this specific set of proteins results in a block to sperm-egg fusion and fertilization. As described in detail in the Examples set forth in Sections 6 and 7 herein below, a number of members of this class of proteins have been identified and characterized. These egg plasma membrane protein antigens are described in detail herein.

The invention is directed to mouse egg plasma membrane polypeptides, herein called M70 polypeptides, that are involved in sperm-egg fusion. The M70 polypeptides of the invention encompass polypeptides which can be isolated from mouse egg which: 1) are located on the egg plasma membrane; 2) have a molecular weight of 70kDa; 3) have a pI of 5; 4) possess a covalently linked glycosylated phosphatidylinositol moiety; and 5) are specifically released from the egg plasma membrane upon treatment with phosphatidylinositol-specific phospholipase C (PI-PLC). Other activities associated with such M70 polypeptides are antigenicity (ability to bind to an anti-M70 antibody or compete with M70 for binding), immunogenicity (ability to generate antibody which binds to M70).

In another aspect, the invention relates to polypeptides identified on the mouse egg plasma membrane, herein called M35/45 polypeptides, that are involved in sperm egg-fusion. The M35/45 polypeptides of the invention encompass polypeptides which can be isolated from mouse egg which: 1) are located on the egg plasma membrane; 2) have a molecular weight of between 35 and 45 kDa; 3) have a pI of 5.5; 4) possess a covalently linked glycosylated phosphatidylinositol moiety; and 5) are specifically released from the egg plasma membrane upon treatment with phosphatidylinositol-specific phospholipase C (PI-PLC). Other activities associated with such M35/45 polypeptides are antigenicity (ability to bind to an anti-M35/45 antibody or compete with M35/45 for binding), immunogenicity (ability to generate antibody which binds to M35/45). In various aspects of the invention the M35/45 polypeptides migrate on polyacrylamide gels as a protein with a molecular weight of 35 kDa, 36kDa, 37 kDa, 38 kDa, 39 kDa, 40 kDa, 41 kDa, 42kDa, 43 kDa, 44 kDa, and 45 kDa,.

In another aspect, the invention relates to polypeptides identified on the hamster egg surface, herein called H25/40 polypeptides, that are involved in sperm egg-fusion. The H25/40 polypeptides of the invention encompass polypeptides which can be isolated from hamster eggs which: 1) are located on the egg plasma membrane; 2) have a molecular weight of between 25 and 40 kDa; 3) have a pI of between 5 and 6; 4) possess a covalently linked glycosylated phosphatidylinositol moiety; and 5) are specifically released from the egg plasma membrane upon treatment with phosphatidylinositol-specific

phospholipase C (PI-PLC). Other activities associated with such H25/40 polypeptides are antigenicity (ability to bind to an anti-H25/40 antibody or compete with H25/40 for binding), immunogenicity (ability to generate antibody which binds to H25/40). In various aspects of the invention the H25/40 polypeptides migrate on polyacrylamide gels as a protein with a molecular weight of between 25 and 30 kDa, or between 30 and 35kDa, or between 36 and 40 kDa. In various aspects of the invention, the H25/40 polypeptides migrate on polyacrylamide gels as a protein with a molecular weight of 25 kDa, 26kDa, 27 kDa, 28 kDa, 29 kDa, 30 kDa, 31 kDa, 32kDa, 33 kDa, 34 kDa, 35 kDa, 36kDa, 37 kDa, 38 kDa, 39 kDa, and 40 kDa.

In another aspect, the invention relates to GPI-linked ZP3 polypeptide, or fragment or analog thereof. ZP3 is a well known, highly conserved, zona pellucida polypeptide. As described herein, a novel form of SP-3 has been found associated with egg membrane. This novel GPI-linked ZP3 is useful as a vaccine for sterilization or contraception of an animal or human subject. An antigenic fragment of GPI-linked ZP3 can comprise a GPI moiety linked to 1-10 amino acids, 10-30 amino acids, 30- 80 amino acids, 50-100 amino acids, 100-200 amino acids, or 200-450 amino acids of ZP3. In various aspects, the invention provides isolated M70, M35/40, and H25/40 polypeptide antigens. In other aspects, the invention provides purified M70, M35/40, and H25/40 polypeptide antigens.

In another aspect of the invention, the GPI-linked ZP3 of the invention encompasses a GPI-linked ZP3 homolog or ortholog from any species. For example, a GPI-linked egg surface protein can comprise (A) the amino acid sequence shown in Fig. 12B (SEQ ID NO:2), or, (B) an amino acid sequence encoded by a DNA sequence that hybridizes to the complement of the DNA sequence of Fig. 12A (SEQ ID NO: 1) under highly stringent conditions, *e.g.*, hybridization to filter-bound DNA in 0.5 M $NaHPO_4$, 7% sodium dodecyl sulfate (SDS), 1 mM EDTA at 65°C, and washing in 0.1xSSC/0.1% SDS at 68°C (Ausubel F.M. *et al.*, eds., 1989, Current Protocols in Molecular Biology, Vol. I, Green Publishing Associates, Inc., and John Wiley & sons, Inc., New York, at p. 2.10.3); and/or (C) an amino acid sequence encoded by DNA sequence that hybridizes to the complement of the DNA sequence of Fig. 12A (SEQ ID No: 1) under less stringent conditions, such as moderately stringent conditions, *e.g.*, washing in 0.2xSSC/0.1% SDS at 42°C (Ausubel *et al.*, 1989, *supra*), and encodes a gene product functionally equivalent to a ZP3 gene product.

The invention further relates to fragments (and derivatives and analogs thereof) of M70, M35/45, H25/40 and GP1-linked ZP3, which comprise one or more domains of an M70, M35/45, or H25/40 protein. In one aspect the M70, M35/45, or

H25/40 fragment is a glycolsylated phosphotidyinositol linked fragment. In another aspect the M70, M35/45, or H25/40 fragment is an extracellular domain fragment.

In one aspect of the invention, egg plasma membrane polypeptides, such as M70, M35/45, H25/40, and GP1-linked ZP3, bind to a sperm protein to facilitate sperm-egg fusion.

Egg plasma membrane polypeptides involved in sperm-egg fusion, such as M70, M35/45, H25/40, and GP1-linked ZP3, or peptide fragments thereof, can be prepared for a variety of uses. For example, such polypeptides, or peptide fragments thereof, can be used for the generation of antibodies, for use in diagnostic and therapeutic assays, or for the identification of agents or small molecules that modulate sperm-egg fusion process required for fertilization.

5.2 PURIFICATION OF EGG SURFACE PROTEINS

Egg plasma membrane polypeptides involved in sperm-egg fusion, such as M70, M35/45, H25/40, and GP1-linked ZP3, can be purified from any cell type that expresses such polypeptides, such as mouse or hamster egg cells. In a preferred embodiment, the M70, M35/40, and H25/40 polypeptides are purified from a fraction of an extract of such cells enriched for cell membrane components. For example, egg plasma membrane polypeptides, such as M70, M35/40, and H25/40 proteins can be solubilized from cells, or cell extracts, such as a cell membrane fraction. The solubilized proteins can subsequently be purified by various procedures known in the art, including but not limited to chromatography (*e.g.*, ion exchange, affinity, and sizing chromatography), centrifugation, electrophoretic procedures, differential solubility, or by any other standard technique for the purification of proteins.

As exemplified by the Examples set forth in Sections 6 and 7, protein "spots" that appear in samples from normal eggs, but are absent in PI-PLC treated eggs can be analyzed further. Differences can be detected by visual inspection of gels, or by using densitometry and computerized image analysis thereby facilitating spot detection, background subtraction and spot matching (*see* Pennington et al., 1997, Trends Cell Biol. *7*: 168-73). Further, egg plasma membrane polypeptides, such as M70, M35/40, or H25/40 protein can be detected by Western Blot analysis of 2D gels, if antibody is available (Harlow and Lane, 1988, *supra*). Once identified, the molecular weight (MW) and the isoelectric point (pI) of an egg surface protein can be determined by calibrating its position relative to known standards run in parallel on 2D gels. Specific proteins can then be purified, and their sequence determined, or a portion of their sequence determined, by

techniques well known in the art, such as Edman degradation sequencing (Edman and Begg, 1967, Eur. J. Biochem. 1:80-91; see, *e.g.*, Creighton, 1983, "Proteins: Structures and Molecular Principles," W.H. Freeman & Co., N.Y., pp.34-49), automated by electroblotting onto polyvinylidene difluoride (PVDF) membranes using Edman degradation chemistry determined by gas-liquid phase, liquid-pulse or solid phase sequence analysis (Findlay and Geisow, 1989, Protein Sequencing: A Practical Approach, IRL Press, Oxford, pp. 1-199). Alternatively, proteins and peptides can be characterized by mass spectrometry, using peptide-mass fingerprinting or protein sequencing methodologies to identify sequence information and post-translational modifications (Dainese et al., 1997, Electrophoresis, 18:432-42; Mann and Wilm, 1995, Trends Biochem. Sci., 20:219-24; Yates, 1996, Methods Enzymol. 271:351-77). After limited sequence information is obtained, protein (Swiss-Prot; http://www.expasy.ch) and nucleic acid sequence (Genebank and EMBL; http://ncbi.nlm.nih.gov) databases can be searched to determine if the protein sequence is novel. Proteins will be analyzed further, used to generate antibodies, as described in Section 5.7, and used for identification of nucleic acid sequences. The amino acid sequence obtained may be used as a guide for the generation of oligonucleotide mixtures that can be used to screen for gene sequences encoding such proteins.

The egg plasma membrane proteins of the present invention can be analyzed by assays based on their physical, immunological, or functional properties. The amino acid sequences of egg plasma membrane polypeptides involved in sperm-egg fusion, such as M70, M35/45, H25/40, and GP1-linked ZP3, can be derived by deduction from the DNA sequence if such is available, or alternatively, by direct sequencing of the protein, *e.g.*, with an automated amino acid sequencer. The protein sequences can be further characterized by a hydrophilicity analysis (Hopp and Woods, 1981, Proc. Natl. Acad. Sci. U.S.A. 78:3824-3828). A hydrophilicity profile can be used to identify the hydrophobic and hydrophilic regions of the protein (and the corresponding regions of the gene sequence, if available, which encode such regions).

Secondary structural analysis (Chou and Fasman, 1974, Biochemistry 13:222) can also be done, to identify regions of the egg surface protein sequence that assume specific secondary structures. Other methods of structural analysis can also be employed. These include but are not limited to X-ray crystallography (Engstom, 1974, Biochem. Exp. Biol. 11:7-13) and computer modeling (Fletterick, R. and Zoller, M. (eds.), 1986, Computer Graphics and Molecular Modeling, in Current Communications in Molecular Biology, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York).

Alternatively, egg plasma membrane polypeptides involved in sperm-egg fusion, such as M70, M35/40, and H25/40 may be isolated and/or purified using

immunological procedures. In one embodiment, where an antibody specific to M70, M35/40, or H25/40 is available, a method of the invention for isolating M70, M35/40, or H25/40 protein comprises the steps of: a) preparing an extract of egg cells; b) contacting a M70, M35/40, or H25/40 specific antibody with the extract for a time period sufficient for the M70, M35/40, or H25/40 in the extract to bind the antibody; and c) recovering the bound antibody. In another embodiment, the method can also be used with an antibody that comprises an affinity tag. Accordingly, the method further comprises incubating the extract and the tagged antibody to the M70, M35/40, or H25/40 protein with a solid phase surface containing a binding partner of the affinity tag for a time period sufficient to allow binding of the egg plasma membrane polypeptides, such as M70, M35/40, or H25/40 to the solid phase surface prior to the recovery step. The isolated protein can then be eluted from the antibody.

Egg plasma membrane polypeptides, such as M70, M35/40, and H25/40 can be purified by isolating cell membranes and purifying the egg plasma membrane polypeptides from other membrane components. Membranes can be isolated from the egg cells of the invention, prepared according to the methods described in Section 6, below. Cells can be lysed and the plasma membrane fraction can be isolated from cells using procedures known in the art, such as dextran/polyethylene glycol biphase separation. Plasma membranes can be treated with a buffer which dissociates membrane-associated proteins from the lipid bilayer (*e.g.*, a buffer containing a non-ionic detergent such as Nonidet P-40™, Triton X-100™, or sodium deoxycholate). Proteins can be purified away from membrane lipids using conventional dialysis procedures.

In one embodiment, the crude dialysed protein preparation can be applied to an affinity column composed of antibody or antiserum stabilized on an appropriate matrix. Membrane-associated proteins other than M70, M35/40, or H25/40 will pass through the column, while M70, M35/40, or H25/40 will remain bound. Non-specific binding of other membrane components can be reduced by increasing the salt concentration and varying the pH of the buffer in which the crude protein preparation is dissolved. Thorough washing of the column after application of the crude protein preparation can further reduce binding of non-specific proteins.

In another embodiment, egg plasma membrane polypeptides, such as M70, M35/40, or H25/40 protein may be purified using specific antibodies, previously generated against M70, M35/40, or H25/40 protein (see Section 5.7, *infra*), or cell membranes. The crude protein preparation from membrane dialysis is applied to an antibody affinity column which is composed of M70, M35/40, or H25/40-specific antibody, stabilized on an appropriate matrix. Antibody-coupled resin, or filter methods can also be used, or other

antibody affinity techniques known in the art (see, for *e.g.*, Harlow and Lane, 1988, *supra*). The column or resin can be washed with buffer to remove proteins which bind non-specifically. The protein which remains bound to the column is eluted by conventional procedure such as washing with a buffer containing high salt or low pH.

In yet another embodiment, egg plasma membrane polypeptides, such as M70, M35/40, or H25/40 protein may be purified by sizing or ion exchange column chromatography. FPLC may be used to facilitate purification of large amounts of protein. If antibody to M70, M35/40, or H25/40 is generated or is available, protein may be detected and followed during purification by Western blot or ELISA (enzyme-linked immunosorbent assay) analysis.

5.3 ISOLATION OF NUCLEIC ACID SEQUENCES

The egg plasma membrane polypeptide nucleotide sequences of the invention can be isolated directly from mRNA, cDNA or from a cDNA or genomic library. Alternatively, egg plasma membrane polypeptide cDNA can be isolated indirectly by first isolating and characterizing the egg plasma membrane polypeptide protein, and subsequently using the egg plasma membrane polypeptide sequence to identify gene sequences in a cDNA or genomic library. Details of such methods are fully described herein.

Nucleic acids encoding egg plasma membrane polypeptides involved in sperm-egg fusion, such as M70, M35/45, H25/40, and GP1-linked ZP3, can be isolated by a variety of methods well known to those of skill in the art, including, but not limited to: screening a cDNA expression library in mammalian cells using egg plasma membrane polypeptide specific antibodies, or "panning"; screening a cDNA expression library in bacterial cells; or differential expression methods such as screening a subtracted cDNA library with an egg plasma membrane polypeptide nucleic acid probe or a specific antibody.

5.3.1 Preparation of mRNA and cDNA

The methods for the purification of mRNA and the synthesis of complementary DNA (cDNA) from egg RNA are described herein. The procedures described in standard treatises, *e.g.*, Methods in Enzymology, 1987, volume 154, Academic Press; Sambrook *et al.*, 1989, Molecular Cloning - A Laboratory Manual, 2nd Edition, Cold Spring Harbor Press, New York; and Ausubel *et al.*, eds., 1992, Current Protocols in Molecular Biology, Greene Publishing Associates and Wiley Interscience, New York, may be followed to carry out routine molecular biology reactions in purification of mRNA.

Methods described in detail *infra* are for illustration only and not by way of limitation. Various mRNA and cDNA preparation systems that are commercially available may also be used according to the manufacturer's instructions for making the mRNA and cDNA of the invention.

Total ribonucleic acid (RNA) may be isolated from egg cells by a variety of methods known in the art depending on the source and amount of cells. It is preferable to obtain good quality RNA that is of high molecular weight in order to construct cDNA libraries that contain even rarely expressed gene products. To prepare high quality RNA, methods that provide complete lysis of cells, and rapid inactivation of nucleases are preferred. A single-step RNA preparation method uses the strong chaotropic agent, guanidinium isothiocyanate, with a mild detergent and 2-mercaptoethanol or dithiothreitol to denature proteins and inactivate nucleases, followed by purification of the RNA by ultracentrifugation (Chomczynski and Sacchi, 1987, Anal Biochem 162:156-159; Chomczynski, 1989, U.S. Patent No. 4,843,155). This procedure may also be used especially when isolating RNA from small quantities of cellular material.

Preferably, total RNA isolated from cells is further purified before conversion into complementary DNA (cDNA). Since the vast majority of eukaryotic messenger RNA (mRNA) molecules contain tracts of poly(adenylic) acid (poly-A) at the 3′ end, it can be enriched by affinity chromatography using oligo-dT cellulose (Aviv and Leder, 1972, Proc. Natl. Acad. Sci., 69:1408-1412). Total RNA is denatured to expose the poly-A tails. Poly-A+ RNA is then bound to oligo-dT cellulose, with the remainder of the RNA washing through. The poly-A+ RNA is eluted by removing salt from the solution. This step may be repeated to further enrich for messenger RNA. A wide variety of oligo-dT matrices in different configurations may also be used, including but not limited to, simple gravity columns, para-magnetic particles, and spin columns. Substituted oligo-dT, such as biotinylated oligo-dT, may also be used. The quantity and quality of RNA thus obtained may be determined by methods such as formaldehyde agarose gel electrophoresis. The use of RNA enriched for poly-A+ RNA is most preferred.

Conversion of RNA into double-stranded cDNA can be accomplished by a number of different procedures well known in the art. See for example, Okayama and Berg, 1982, Mol. Cell Biol. 2:161-170; Gubler and Hoffman, 1983, Gene 25:263-269; and Huse and Hansen, 1988, Strategies (Stratagene) 1:1-3. The first step in the making of cDNA involves the oligonucleotide-primed synthesis of a first strand cDNA by reverse transcriptase. For example, mRNA hybridized to an oligo-dT primer can be copied into DNA by a reverse transcriptase, such as AMV reverse transcriptase, MMLV reverse transcriptase, or Superscript (Kotewicz *et al.*, 1988, Nucleic Acid Res. 16:265-277).

Random hexamers may be used to prime first-strand synthesis from internal sites within the mRNA instead of oligo-dT primers resulting in shorter cDNAs which are enriched for the 5' ends of long messenger RNAs.

The next step in the process involves synthesizing the second strand cDNA and producing suitable DNA ends for insertion in a cloning vector. Briefly, for example, the second strand cDNA may be synthesized using E. coli DNA polymerase I, Klenow fragment using the RNA-DNA as a template. The RNA in the RNA-DNA hybrid can be removed with RNase H, and gaps in the newly synthesized second strand cDNA can be filled in by E. coli DNA polymerase I. The fragments of second strand cDNAs thus produced are ligated with E. coli DNA ligase to form a contiguous second strand cDNA.

After second strand DNA synthesis, the double stranded cDNA requires further repair with enzymes, such as RNase H, RNase A, T4 DNA polymerase and E. coli DNA ligase, to form perfectly matched strands (*i.e.*, having "flush" or "blunt" ends).

In some protocols, where the amount of starting cellular material is very limited, the cDNA can be amplified *in vitro*, by nucleic acid amplification methods known in the art, such as polymerase chain reaction (PCR) and ligation-mediated chain reaction (LCR). Generally, first strand oligo-dT primed cDNA obtained by a standard method is extended with a oligo-dG tail by terminal transferase, and a second primer containing a oligo-dC segment is used to prime second strand synthesis with a thermostable DNA polymerase. This procedure produces a double-stranded cDNA population each molecule of which is bracketed by two oligonucleotides of known sequence. Using the appropriate set of primers, standard PCR can be used to amplify the cDNA. See, for example, U.S. Patent Nos. 4,683,202, 4,683,195 and 4,889,818; Gyllenstein *et al.*, 1988, Proc. Natl. Acad. Sci. USA 85:7652-7656; Ochman *et al.*, 1988, Genetics 120:621-623; Loh *et al.*, 1989, Science 243:217-220; Tam *et al.*, 1989, Nucleic Acid Res. 17:1269; Belyavsky *et al.*, 1989, Nucleic Acid Res. 17:2919-2932. In specific embodiments of the invention, RT-PCR can be used to generate amplified cDNAs from the RNAs (see, *e.g.*, Domec *et al.*, 1990, Anal Biochem, 188:422-426; Van Gelder *et al.*, 1990, Proc. Natl. Acad. Sci., 87:1663-1667).

In another embodiment, the egg plasma membrane polypeptide nucleic acid sequences can be isolated by identifying genes that are expressed in eggs but not in other cell types (such as sperm or somatic cells). A number of methods exist for identifying such differentially expressed genes between two or more cell types. For example, differential display of cDNA 3' end sequences (Liang and Pardee, 1992, Science 257:967-971), serial analysis of gene expression by comparative gels of PCR products (SAGE; Velculescu et al., 1995, Science 270:484-487), or nucleic acid array (DNA chip) technology (Schena et al.,

1995, Science 270:467-470; see also, J. Ramsey, 1998, Nat. Biotechnology 1:40-44), can be used to identify differentially expressed genes in a non-selective manner.

For example, differential display can be used identify egg plasma membrane polypeptide cDNA sequences present in egg cells but absent in control cells. Egg cells and negative control cells are prepared, and mRNA is isolated as described, *supra*. cDNA is prepared from mRNA, as described, *supra*, using RT-polymerase chain amplification with a set of labeled oligonucleotide primers designed to identify the 3' ends of mRNAs (Liang and Pardee, 1992, *supra*). Primers used for the synthesis of the first strand each contain a stretch of oligo dT at its 5' end, followed by a pair of nucleotides at its 3' end. Such oligonucleotides are end-labeled and used as primers in reverse-transcriptase polymerase chain reactions (RT-PCR) to generate a population of specific cDNAs. Products of such RT-PCR reactions are displayed on a sequencing gel. Using mRNAs derived from different populations of cells, the pattern of displayed products can be compared to identify bands that are unique to different cell types (Liang and Pardee, 1995, Curr. Opin. Immunol., 7:274-280; McClelland, M. et al., 1995, Trends Genet., 11, 242-246).

mRNA from eggs is compared to mRNA from control cells by differential display. Following RT-PCR using the specific set of primers described hereinabove, RT-PCR products are displayed on thin polyacrylamide gels containing 8% urea, the type used for DNA sequencing analysis. Products that are detected in egg cells but absent in control cells are chosen to be analyzed further. Gel purification and sequence analysis of such products can be performed to identify egg plasma membrane polypeptide nucleic acid candidates. Protein-coding sequences of egg plasma membrane polypeptide candidates, i.e., sequences present in egg cells but not in control cells, can be compared to known protein sequences in a data base such as Swiss-prot (Bairoch and Apweiler, 1998, Nucl. Acids Res. 26:38-42; http://www.expasy.ch). Novel sequences can be chosen as potential egg plasma membrane polypeptide candidates. Such gene products can then be isolated from the cDNA population using standard cloning techniques (Ausubel *et al.*, eds., 1992, Current Protocols in Molecular Biology, Greene Publishing Associates and Wiley Interscience, New York), and can be tested for their ability to bind egg plasma membrane polypeptide antibodies.

In another embodiment, nucleic acid array technology can be used to identify egg plasma membrane protein-specific sequences. Such micro-arrays of cDNA probes have been successfully used to compare the expression patterns of different cell types (DeRisi, *et al.*, 1996, Nat. Genet.14:457-460). Micro-arrays typically have many different DNA molecules fixed at defined "addresses" on a two dimensional, usually glass, support. Each address contains either many copies of a single DNA, or a mixture of different DNA molecules, and each DNA molecule is usually 1000 nucleotides or less in length. The

DNAs can be from any source, cDNA libraries, or can be synthesized oligonucleotides. A vast excess of probe is fixed at each address, so that the hybridization signal intensity at that address is limited by the concentration of labeled complementary sequence in immediate proximity to the address. The probe array is useful for measuring the ratio of hybridization between to differently labeled samples that are thoroughly mixed and therefore share the same hybridization conditions. Simple probe arrays are currently able to detect cDNA species that are present at 2 to 10 copies of mRNA per cell when contacted with a solution containing a total cDNA concentration of 1 mg/ml. In a preferred embodiment, mRNA derived from egg plasma membrane polypeptide positive hybrid cells and control cells is labeled with distinct fluorophores and hybridized to DNA on a micro-array in a mixture. The sequences of differentially expressed nucleic acids are determined by identifying the addresses where differential hybridization between the two cell populations occurs. These nucleic acid sequences can then be used to identify egg plasma membrane polypeptide gene sequences, to synthesize recombinant protein, and to generate antibodies.

cDNAs can be inserted into an appropriate cloning vector, and introduced into an appropriate host organism for propagation. Such cDNA libraries may then be used for preparation of "subtracted" cDNA libraries, or for direct and expression screening for egg plasma membrane polypeptide gene sequences. Methods for such procedures are well known in the art, and are described in standard treatises, *e.g.*, Methods in Enzymology, 1987, volume 154, Academic Press; Sambrook *et al.*, 1989, Molecular Cloning - A Laboratory Manual, 2nd Edition, Cold Spring Harbor Press, New York; and Ausubel *et al.*, eds., 1992, *supra.*

Selective protocols can be used to specifically increase the abundance of sequences overexpressed in one population relative to another by elimination of gene products common for both from the two populations by means of subtractive hybridization. A number of such subtraction hybridization protocols can be used, including, but not limited to, representational difference analysis (Fargnoli et al., 1990, Anal. Biochem., 187:364-73; Wang and Brown, 1991, Proc. Natl. Acad. Sci. 88:11505-09; see Lisitsyn, 1995, Trends Genet. 11:303-7), enzymatic degrading subtraction (EDS; Zeng *et al.*, 1994, Nuc. Acid Res. 22:4381-85), RecA-mediated subtraction hybridization (Hakvoort *et al.*, 1996, Nucl. Acids Res. 24:3478-80) or selective amplification via biotin and restriction mediated enrichment (SABRE; Lavery *et al.*, 1997, Proc. Natl. Acad. Sci. USA 13:6831-36). Briefly, a subtracted library is prepared from eggs and negative control cells. The egg cells and control cells are prepared, and egg cDNA is hybridized to a large excess of poly A+ mRNA from control cells. cDNA molecules expressed only in eggs will not hybridize, and can be removed by passing mixture over a hydroxylapatite column under conditions

such that the column specifically retains RNA:DNA duplexes but not DNA or DNA duplexes. The column flow-through, containing cDNAs representing mRNAs that are expressed in eggs but not in negative control cells, is cloned into an appropriate vector.

In another embodiment, biotinylated RNA or cDNA can be used to label negative control cells. After hybridization, the egg specific sequences can be eliminated by passing the hybridization mixture over a streptavidin column. The flow-through can be cloned into an appropriate expression vector. In another embodiment, any of the above mentioned techniques for subtractive hybridization techniques can be used, or any of the other techniques known in the art. The library thus created is plated out to isolate single colonies. In one embodiment, since this cDNA library contains egg-specific sequences that are absent in negative control cells, the cDNA inserts can simply be sequenced at this point. In another embodiment, the colonies can be transferred to nitrocellulose filters. The filters can be incubated with antibody specific to egg plasma membrane polypeptide, and the antibody detected using methods known in the art. In yet another embodiment, the colonies can be transferred to nitrocellulose filters, and incubated, under conditions that promote nucleic acid hybridization, with a nucleic acid probe. For example, if egg plasma membrane polypeptide sequence information is available, a labeled degenerate oligonucleotide can be designed. Protein sequence information is used to design degenerate oligonucleotides containing all possible codons for egg plasma membrane polypeptide amino acids. Sequence information from various regions of the protein can be used to generate a series of such degenerate pools of oligonucleotides, where each oligonucleotide pool contains some sequences that are complementary to egg plasma membrane polypeptide gene sequences. Such degenerate oligonucleotide pools can be used to screen the subtracted cDNA library, cDNA inserts can be sequenced, and sequence of positive clones can be used to screen a genomic library and thus identify egg plasma membrane polypeptide genes.

The cDNA inserts can be sequenced, and the sequence of positive clones can be used to screen a genomic library and thus identify egg plasma membrane polypeptide gene.

The present invention provides various methods for isolation of nucleic acid molecules encoding egg plasma membrane polypeptide by screening cDNA and/or genomic DNA library. A gene library comprises a pool of nucleic acid molecules, in which one or more nucleic acid molecules comprise nucleotide sequences encoding egg plasma membrane polypeptide or a fragment thereof. A gene library can be introduced into the appropriate recombinant cells for replication and screening and for production of the proteins encoded by the cDNAs.

In one embodiment, the invention provides a method for screening a gene library for the egg plasma membrane polypeptide gene using one or more nucleic acid probe, such as a pool of degenerate oligonucleotides having sequences that encode egg plasma membrane polypeptide or a fragment thereof. The nucleic acid sequence of the probe can be designed in accordance to available peptide sequence of an egg plasma membrane polypeptide, a fragment or homolog thereof. For example, a probe based on the egg plasma membrane polypeptide peptide sequence of one species can be used to identify and isolate the egg plasma membrane polypeptide gene of a related species. Egg plasma membrane polypeptide can be purified and sequenced. Protein sequence information is then used to design degenerate oligonucleotides containing all possible codons for egg plasma membrane polypeptide amino acids. Sequence information from various regions of the protein can be used to generate a series of such degenerate pools of oligonucleotides. Thus, each oligonucleotide pool contains some sequences that are complementary in its entirety to egg plasma membrane polypeptide gene sequences. Such degenerate oligonucleotide pools can be used to screen a gene library, prepared as described herein, supra. Accordingly, the method comprises (a) incubating a labeled nucleic acid probe with DNA molecules derived from recombinant cells containing a plurality of DNA molecules from egg plasma membrane polypeptide positive hybrid cells, for a time period sufficient to allow hybridization of the labeled probe to the DNA molecules, wherein the labeled probe having a nucleic acid sequence that comprises a sequence that encodes egg plasma membrane polypeptide or a fragment thereof; (b) identifying the recombinant cell containing the DNA molecule to which the labeled probe bound; (c) recovering the DNA molecule present in the recombinant cell.

In another embodiment, the invention provides methods for identifying and isolating the egg plasma membrane polypeptide gene that rely on expression of cDNA insert and screening for its activity by binding assays, immunological methods, or an altered cellular phenotype. The egg plasma membrane polypeptide cDNA can be isolated indirectly by screening the cDNA expression library for egg plasma membrane polypeptide activity, such as egg plasma membrane polypeptide antibody-binding activity. For example, egg plasma membrane polypeptide antibodies can be labeled with a detectable compound, such as a radioactive, fluorescent or biotinylated compound, and used as probes to screen bacterial colonies that have been induced to express cDNA inserts.

A eukaryotic expression library can be screened by "panning" (Seed, 1987, Proc. Natl. Acad. Sci. USA 84:3365-69). This method is particularly preferred for screening cDNA molecules encoding proteins that are expressed on the cell surface. Using this technique, culture dishes are pre-coated with antibody, which can bind to cells that

express egg plasma membrane polypeptide. Alternatively, culture dishes may be coated with an egg plasma membrane polypeptide ligand, which also can bind to cells that express egg plasma membrane polypeptide. Non-adherent cells can be rinsed away, and selected cells can be isolated and their inserts can be further analysed. It is preferable that the type of host cell used in panning is non-adherent to surfaces of cell culture containers, such as plastic, so as to facilitate the screening methods of the invention. In one embodiment, an SV40 vector and control sequences are utilized, and the resulting cDNA library is introduced into African green monkey cells (COS cells). The cDNA library can be constructed in a vector containing viral control regions, and introduced in mammalian cells by transfection or infection with viral vectors. Cells are distributed on microtiter dishes for screening. The cDNA library can be transiently expressed in mammalian cells. In a preferred embodiment, the cDNA used in constructing the library is prepared from mRNA isolated from the egg plasma membrane polypeptide positive hybrid cells of the invention. In another embodiment, the library is a subtracted cDNA library, wherein gene products common to both egg plasma membrane polypeptide positive hybrid cells and egg plasma membrane polypeptide negative hybrid cells are eliminated from the egg plasma membrane polypeptide positive hybrid cell mRNA or cDNA population by means of subtractive hybridization prior to construction of the cDNA library. In yet another embodiment, the library is an cDNA library, or a "subtracted" cDNA library, in which cDNAs common to both egg and sperm, or a somatic cell, are subtracted from egg cDNA population prior to cloning (Fargnoli et al., 1990, Anal. Biochem., 187:364-73; Wang and Brown, 1991, Proc. Natl. Acad. Sci. 88:11505-09; Lisitsyn, 1995, Trends Genet. 11:303-7).

An expression construct, as used herein, refers to a polynucleotide comprising egg plasma membrane polypeptide positive hybrid cell derived cDNA sequences operably associated with one or more regulatory regions which enables expression of the library of cDNAs in an appropriate host cell. "Operably-associated" refers to an association in which the regulatory regions and the cDNA sequence to be expressed are joined and positioned in such a way as to permit transcription, and ultimately, translation.

The regulatory regions necessary for transcription of the cDNA library can be provided by an expression construct. A translation initiation codon (ATG) may also be provided if the cDNA fragments without their cognate initiation codon are to be expressed. In a compatible host-construct system, cellular transcriptional factors, such as RNA polymerase, will bind to the regulatory regions on the expression construct to effect transcription of the cDNA library in the host organism. The precise nature of the regulatory regions needed for gene expression may vary from host cell to host cell. Generally, a

promoter is required which is capable of binding RNA polymerase and promoting the transcription of an operably-associated nucleic acid sequence. Such regulatory regions may include those 5'-non-coding sequences involved with initiation of transcription and translation, such as the TATA box, capping sequence, CAAT sequence, and the like. The non-coding region 3' to the coding sequence may contain transcriptional termination regulatory sequences, such as terminators and polyadenylation sites. In order to be "operably-associated", it is not necessary that the regulatory region and the cDNA sequences be immediately adjacent to one another. Regulatory regions suitable for gene expression are well known in the art (see Section 5.6).

Both constitutive and inducible regulatory regions may be used for cDNA expression. It may be desirable to use inducible promoters when the conditions optimal for growth of the host cells and the conditions for high level expression of the cDNA library are different. This use of an inducible regulatory region may be particularly desirable if some of the proteins encoded by the cDNAs confer growth advantages or disadvantage to the recombinant host cells expressing them. Examples of useful regulatory regions are provided in the next section below.

The expression constructs comprising the cDNA library operably associated with regulatory regions can be directly introduced into appropriate host cells. See, for example, U.S. Patent No. 5,580,859. The expression constructs can also comprise at both ends specific oligonucleotide sequences, which may be utilized as primers to amplify the cDNAs by polymerase chain reaction (PCR). The design of the primer sequences for DNA amplification and the ligation of the primer sequences to the cDNAs can be carried out by any methods known in the art, including those described above employing linkers and adaptors. The amplification can be carried out, *e.g.*, by use of a Perkin-Elmer Cetus thermal cycler and Taq polymerase (Gene Amp™). Such a library of cDNA expression constructs can be amplified and maintained *in vitro*, without the use of DNA sequences that propagate the polynucleotide within living cells. Depending on needs, an aliquot of the cDNA expression library can be thawed and introduced directly into host cells. Such expression constructs can be used for expression of cDNAs transiently in recombinant host cells.

5.3.2 cDNA Expression Cloning

Described herein are systems of vectors and host cells that can be used for cloning and expression of a cDNA library. An expression vector is a cloning vector that can be used for maintenance and expression of cDNA library in an appropriate host cell. Any cloning vector known in the art can be used to propagate the cDNA library. A variety of cloning vectors may be used in the present invention which include, but are not limited

to, plasmids, cosmids, phage, phagemids, or modified viruses. Typically, such cloning vectors comprise a functional origin of replication for propagation of the vector in an appropriate host cell, one or more restriction endonuclease sites for insertion of the cDNA library, and one or more selection markers. The cloning vector must be used with a compatible host cell which may be derived from a prokaryotic or an eukaryotic organism including but not limited to bacteria, yeasts, insects, mammals, and humans.

Expression constructs and vectors are introduced into host cells for the purpose of expressing the cDNA library. Host cells broadly encompass cells of unicellular organisms, such as bacteria, fungi, and yeast, and of multicellular organisms, such as insects and animals including but not limited to birds, mammals and humans. Host cells may be obtained from private laboratory deposits, public culture collections such as the American Type Culture Collection, or from commercial suppliers.

cDNA expression cloning in a eukaryotic host is advantageous because egg plasma membrane polypeptide(s) can be post-translationally modified and correctly inserted into the plasma membrane. Such modifications (*e.g.*, glycosylation) and processing (*e.g.*, cleavage) of cDNA-encoded proteins may enhance egg plasma membrane polypeptide activity. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins. A eukaryotic host-vector system is preferred, a mammalian host-vector system is more preferred, and a human host-vector system is the most preferred. Preferred mammalian host cells include but are not limited to those derived from humans, monkeys and rodents, such as chinese hamster ovary (CHO) cells, NIH/3T3, COS, HeLa, Daudi, 293, 293-EBNA, VERO, etc. (see, for example, Kriegler M. in "Gene Transfer and Expression: A Laboratory Manual", New York, Freeman & Co. 1990).

Any of the cloning and expression vectors described herein may be synthesized and assembled from known DNA sequences by well known techniques in the art. The regulatory regions and enhancer elements can be of a variety of origins, both natural and synthetic. Some vectors and host cells may be obtained commercially. Non-limiting examples of useful vectors are pCDM8, λDR2 (see Ausubel *et al.*, eds.,1988, Current Protocols in Molecular Biology, Greene Publishing Associates and Wiley Interscience, New York, Appendix, which is incorporated herein by reference). By way of example, an exemplary expression host-vector system is λDR2 which is a lambda bacteriophage-based cloning vector coupled with a mammalian expression plasmid. Advantages of this system include the utilization of highly efficient lambda *in vitro* packaging systems for initially generating a library in E. coli hosts. Size selection may not be required since the packaging system only accepts inserts in a certain size range. Lambda

vectors generally provide greater ease in amplification and storage. The initial library in E. coli may be amplified to produce supercoiled plasmid DNA which may be used in high efficiency transformation methods for introduction into other expression host organisms. For example, λDR2 uses the lox P mediated site-specific recombination to excise the expression vector pDR2 containing a cDNA insert from lambda clones which can recircularize to generate a plasmid. The plasmid pDR2 contains eukaryotic regulatory regions based on the Epstein-Barr virus and selection markers that allows direct introduction of the cDNA inserts as a library into permissive human host cells at high efficiency.

For expression of cDNAs in mammalian host cells, a variety of regulatory regions can be used, for example, the SV40 early and late promoters, the cytomegalovirus (CMV) immediate early promoter, and the Rous sarcoma virus long terminal repeat (RSV-LTR) promoter. Inducible promoters that may be useful in mammalian cells include but are not limited to those associated with the metallothionein II gene, mouse mammary tumor virus glucocorticoid responsive long terminal repeats (MMTV-LTR), β-interferon gene, and Hsp70 gene (Williams *et al.*, 1989, Cancer Res. 49:2735-42 ; Taylor *et al.*, 1990, Mol. Cell Biol., 10:165-75). The efficiency of cDNA expression in a host cell may be enhanced by the inclusion of appropriate transcription enhancer elements in the expression vector, such as those found in SV40 virus, Hepatitis B virus, cytomegalovirus, immunoglobulin genes, metallothionein, β-actin (see Bittner *et al.*, 1987, Methods in Enzymol. 153:516-544; Gorman, 1990, Curr. Op. in Biotechnol. 1:36-47).

In addition, the expression vector may contain selectable or screenable marker genes for initially isolating, identifying or tracking host cells that contain egg plasma membrane polypeptide cDNA. A number of selection systems may be used for mammalian cells, including but not limited to the Herpes simplex virus thymidine kinase (Wigler *et al.*, 1977, Cell 11:223), hypoxanthine-guanine phosphoribosyltransferase (Szybalski and Szybalski, 1962, Proc. Natl. Acad. Sci. USA 248:2026), and adenine phosphoribosyltransferase (Lowy *et al.*, 1980, Cell 22:817) genes can be employed in tk^-, $hgprt^-$ or $aprt^-$ cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for dihydrofolate reductase (dhfr), which confers resistance to methotrexate (Wigler *et al.*, 1980, Natl. Acad. Sci. USA 77:3567; O'Hare *et al.*, 1981, Proc. Natl. Acad. Sci. USA 78:1527); gpt, which confers resistance to mycophenolic acid (Mulligan and Berg, 1981, Proc. Natl. Acad. Sci. USA 78:2072); neomycin phosphotransferase (neo), which confers resistance to the aminoglycoside G-418 (Colberre-Garapin *et al.*, 1981, J. Mol. Biol. 150:1); and hygromycin phosphotransferase (hyg), which confers resistance to

hygromycin (Santerre *et al.*, 1984, Gene 30:147). Other selectable markers, such as but not limited to histidinol and Zeocin™ can also be used.

A number of viral-based expression systems may also be utilized with mammalian cells to make the cDNA libraries. Vectors using DNA virus backbones have been derived from simian virus 40 (SV40) (Hamer *et al.*, 1979, Cell 17:725), adenovirus (Van Doren *et al.*, 1984, Mol Cell Biol 4:1653), adeno-associated virus (McLaughlin *et al.*, 1988, J. Virol. 62:1963), and bovine papillomas virus (Zinn *et al.*, 1982, Proc Natl Acad Sci 79:4897). In cases where an adenovirus is used as an expression vector, the donor DNA sequence may be ligated to an adenovirus transcription/translation control complex, *e.g.*, the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by *in vitro* or *in vivo* recombination. Insertion in a non-essential region of the viral genome (*e.g.*, region E1 or E3) will result in a recombinant virus that is viable and capable of expressing heterologous products in infected hosts. (See *e.g.*, Logan and Shenk, 1984, Proc. Natl. Acad. Sci. (USA) 81:3655-3659). Alternatively, the vaccinia 7.5K promoter may be used (see, *e.g.*, Mackett *et al.*, 1982, Proc. Natl. Acad. Sci. (USA) 79:7415-7419; Mackett *et al.*, 1984, J. Virol. 49:857-864; Panicali *et al.*, 1982, Proc. Natl. Acad. Sci. 79:4927-4931.) In cases where a human host cell is used, vectors based on the Epstein-Barr virus (EBV) origin (OriP) and EBV nuclear antigen 1 (EBNA-1; a trans-acting replication factor) can be used. Such vectors can be used with a broad range of human host cells, *e.g.*, EBO-pCD (Spickofsky *et al.*, 1990, DNA Prot Eng Tech 2:14-18); pDR2 and λDR2 (available from Clontech Laboratories). The expression vector pDR2 carries the EBV origin which confers stable episomal maintenance to the vector when activated by EBNA-1. Extremely high transfection efficiencies up to 10^{-1} can be obtained when pDR2 is transfected into cell lines which express EBNA-1. Host cells can be rendered proficient for high-efficiency transfections by first transfecting the host cells with an expression construct that produces EBNA-1.

cDNA libraries may also be made with a retrovirus-based expression cloning system. Retroviruses, such as Moloney murine leukemia virus, can be used since most of the viral gene sequence can be removed and replaced with the cDNA library while the missing viral functions can be supplied in trans. In contrast to transfection, retroviruses can efficiently infect and transfer genes to a wide range of cell types including, for example, primary hematopoietic cells. Moreover, the host range for infection by a retroviral vector can be manipulated by the choice of envelope used for vector packaging.

For example, a retroviral vector can comprise a 5' long terminal repeat (LTR), a 3' LTR, a packaging signal, a bacterial origin of replication, and a selectable marker. The cDNA is inserted into a position between the 5' LTR and 3' LTR, such that

transcription from the 5' LTR promoter transcribes the cloned cDNA. The 5' LTR comprises a promoter, including but not limited to an LTR promoter, an R region, a U5 region and a primer binding site, in that order. Nucleotide sequences of these LTR elements are well known in the art. A heterologous promoter as well as multiple drug selection markers may also be included in the expression vector to facilitate selection of infected cells. See, McLauchlin *et al.*, 1990, Prog. Nucleic Acid Res. and Molec. Biol. 38:91-135; Morgenstern *et al.*, 1990, Nucleic Acid Res. 18:3587-3596; Choulika *et al.*, 1996, J Virol 70:1792-1798.

Other useful eukaryotic host-vector system may include yeast and insect systems. In yeast, a number of vectors containing constitutive or inducible promoters may be used with *Saccharomyces cerevisiae* (baker's yeast), *Schizosaccharomyces pombe* (fission yeast), *Pichia pastoris*, and *Hansenula polymorpha* (methylotropic yeasts). For a review see Ausubel *et al.*, eds., 1989, Current Protocols in Molecular Biology, Greene Publishing Associates and Wiley Interscience, New York, Ch. 13; Grant *et al.*, 1987, Expression and Secretion Vectors for Yeast, in Methods in Enzymology, Eds. Wu and Grossman, 1987, Acad. Press, N.Y., Vol. 153, pp. 516-544; Glover, 1986, DNA Cloning, Vol. II, IRL Press, Wash., D.C., Ch. 3; and Bitter, 1987, Heterologous Gene Expression in Yeast, Methods in Enzymology, Eds. Berger and Kimmel, Acad. Press, N.Y., Vol. 152, pp. 673-684; and The Molecular Biology of the Yeast *Saccharomyces*, 1982, Eds. Strathern *et al.*, Cold Spring Harbor Press, Vols. I and II.

In an insect system, Autographa californica nuclear polyhydrosis virus (AcNPV) a baculovirus, can be used as a vector to express cDNA in Spodoptera frugiperda cells. The cDNA sequences may be cloned into non-essential regions (for example the polyhedrin gene) of the virus and placed under control of an AcNPV promoter (for example the polyhedrin promoter). These recombinant viruses are then used to infect host cells in which the inserted DNA is expressed (see, *e.g.*, Smith *et al.*, 1983, J Virol 46:584; Smith, U.S. Patent No. 4,215,051.)

The recombinant host cells may be cultured under standard conditions of temperature, incubation time, optical density, and media composition.

Expression constructs containing cloned cDNA can be introduced into the host cell by a variety of techniques known in the art, including but not limited to, for prokaryotic cells, λ-phage packaging and infection, bacterial transformation (Hanahan, 1985, in DNA Cloning, A Practical Approach, 1:109-136), and for eukaryotic cells, calcium phosphate mediated transfection (Wigler *et al.*, 1977, Cell 11:223-232), liposome-mediated transfection (Schaefer-Ridder *et al.*, 1982, Science 215:166-168), electroporation (Wolff *et*

al., 1987, Proc. Natl. Acad. Sci. 84:3344), and microinjection (Cappechi, 1980, Cell 22:479-488).

In a specific embodiment, and ovarian-derived cell line may be used. It is preferable that the type of host cell is non-adherent to surfaces of cell culture containers, such as plastic, so as to facilitate screening methods and harvesting of the cells.

In one embodiment, an SV40 vector and control sequences are utilized, and the resulting cDNA library is introduced into African green monkey cells (COS cells). The cDNA library can be constructed in a vector containing viral control regions, and introduced in mammalian cells by transfection or infection with viral vectors. Cells are distributed on microtiter dishes for screening. The cDNA library can be transiently expressed in mammalian cells. In a preferred embodiment, the cDNA used in constructing the library is prepared from mRNA isolated from the egg plasma membrane polypeptide positive hybrid cells of the invention. In another embodiment, the library is a "subtracted" cDNA library, wherein gene products common to both egg plasma membrane polypeptide positive hybrid cells and egg plasma membrane polypeptide negative hybrid cells are eliminated from the egg plasma membrane polypeptide positive hybrid cell mRNA or cDNA population by means of subtractive hybridization prior to construction of the cDNA library (see Section 5.4.4). In yet another embodiment, the library is a ovarian cell cDNA library, or a subtracted ovarian cell library (Fargnoli *et al.*, 1990, Anal. Biochem., 187:364-73; Wang and Brown, 1991, Proc. Natl. Acad. Sci. 88:11505-09; Lisitsyn, 1995, Trends Genet. 11:303-7; Zeng *et al.*, 1994, Nuc. Acid Res. 22:4381-85; Hakvoort *et al.*, 1996, Nucl. Acids Res. 24:3478-80; Lavery *et al.*, 1997, Proc. Natl. Acad. Sci. USA 13:6831-36).

The cDNA library can be screened directly or indirectly. A number of indirect methods are possible that rely on expression of cDNA insert and screening for its activity by binding assays, immunological methods, or an altered cellular phenotype. egg plasma membrane polypeptide can be isolated indirectly by screening the cDNA expression library for egg plasma membrane polypeptide activity, such as ligand binding or egg plasma membrane polypeptide antibody-binding activity. For example, egg plasma membrane polypeptide antibodies can be labeled with a detectable compound, such as a radioactive, fluorescent or biotinylated compound, and used as probes to screen bacterial colonies that have been induced to express cDNA inserts.

In one embodiment, the eukaryotic expression library can be screened by panning (Seed, 1987, Proc. Natl. Acad. Sci. USA 84:3365-69). Using this technique, culture dishes are pre-coated with antibody, which can bind to cells that express egg plasma membrane polypeptide. Alternatively, culture dishes may be coated with egg plasma membrane polypeptide protein, which also can bind to cells that express egg plasma

membrane polypeptide. Non-adherent cells can be rinsed away, and selected cells can be isolated and their inserts can be further analyzed. Alternatively, a cDNA library can be screened directly by hybridization. An oligonucleotide probe designed from the sequence of the egg plasma membrane polypeptide protein. Egg plasma membrane polypeptide(s) can be purified and sequenced, as described in Section 5.2, *supra*. Protein sequence information is then used to design degenerate oligonucleotides containing all possible codons for egg plasma membrane polypeptide amino acids. Sequence information from various regions of the protein can be used to generate a series of such degenerate pools of oligonucleotides. Thus, each oligonucleotide pool contains some sequences that are complementary to egg plasma membrane polypeptide gene sequences. Such degenerate oligonucleotide pools can be used to screen a cDNA library, prepared as described herein, *supra*.

In another embodiment, the expression library can be screened with fluorescently or magnetically labeled egg plasma membrane polypeptide antibody, using cell sorting methods known in the art. Such labeling and sorting methods are described in detail in Section 5.2, *supra*.

For cDNA expression in prokaryotic cells, cDNA can be cloned into a plasmid or phage vector. Vectors based on *E. coli* are the most popular and versatile systems for high level expression of foreign proteins (Makrides, 1996, Microbiol. Rev., 60:512-538). Non-limiting examples of regulatory regions that can be used for expression in E. coli may include but not limited to lac, trp, lpp, phoA, recA, tac, T3, T7 and λP_L (Makrides, 1996, Microbiol Rev, 60:512-538). Non-limiting examples of prokaryotic expression vectors may include the λgt vector series such as λgt11 (Huynh *et al.*, 1984 in "DNA Cloning Techniques", Vol. I: A Practical Approach (D. Glover, ed.), pp. 49-78, IRL Press, Oxford), and the pET vector series (Studier *et al.*, 1990, Methods Enzymol., 185:60-89). λgt11 is particularly advantageous for this purpose. The phage contains the temperature sensitive repressor λc1857 which is inactive at 42°C and the expression of the cDNA insert is under the control of the lac operon (Young and Davis, 1983, Science 222:778-782). Proteins may be induced by shifting temperature to 42°C. In this way, the expression of foreign proteins which are potentially deleterious or lethal to cell growth can be tightly controlled while bacterial colonies are growing at 37°C. Furthermore, in this system, cloning of cDNA insert interrupts the β-galactosidase gene, so that recombinants can be readily identified by addition of the gratuitous *lac* operon inducer isopropyl thio-β-D-galactopyranoside (IPTG) and assaying for β-galactosidase activity, by methods well known in the art, such as plating on X-gal.

Expression constructs containing cloned cDNA can be introduced into the prokaryotic host cell by a variety of techniques known in the art, including but not limited to, λ-phage packaging and infection, transduction and transformation (Hanahan, 1985, in DNA Cloning, A Practical Approach, 1:109-136). Bacteria is infected with phage or transformed with plasmid carrying the cDNA library, plated on LB agar plates, and induced to express cDNA inserts.

However, a potential drawback of a prokaryotic host-vector system is the inability to perform many of the post-translational processing, folding and insertion into membranes normally required of cell surface receptors.

A specific cDNA insert can be detected and isolated by inducing expression of the cDNA inserts and utilizing screening methods that rely on detection of protein activity. Such methods include filter binding to a labeled ligand or immunological methods to detect antibody binding.

For example, in a preferred embodiment, egg surface polypeptide can be isolated by screening the cDNA expression library for egg surface polypeptide activity, such as egg surface polypeptide antibody-binding or ligand binding. For example, egg surface polypeptide antibodies can be labeled with a detectable compound, such as a radioactive, fluorescent or biotinylated compound can be used as probes to screen induced proteins colonies attached to filters. Egg surface polypeptide antibody mixture is incubated using conditions that promote binding and developed as described above to detect egg surface polypeptide clones. Alternatively, immunological methods are used to detect antibody.

In another embodiment, a cDNA library can be screened for egg surface polypeptide expression in frog oocytes. Frog oocytes are advantageous for this purpose because their large size and (1-1.2 mm) and their abundance of protein translation machinery. In addition, insertion of receptor proteins can be inserted into membranes readily screened for activity. A cDNA library is constructed in a vector containing T3, T7, SP6 or other RNA polymerase promoter located on either side of a polylinker containing cloning sites for insertion of cDNA. cDNAs can be prepared and inserted into the vector, the library is amplified, and plasmid DNA is isolated and linearized by cutting with a restriction endonuclease whose site is in the polylinker. Run-off in transcriptions are performed *in vitro*, by addition of nucleotides and the appropriate polymerase, and mRNAs are injected into oocytes. After allowing for translation, oocytes are incubated with ligand labeled with radioactive, fluorescent, or otherwise detectable compound. Sublibraries displaying a positive signal are further divided, plasmid DNA is isolated, *in vitro* transcribed and injected until a single clone is isolated.

Any of the above described methods can be used to identify egg surface polypeptide gene candidates. Positive clones can be isolated, purified and the sequence of their inserts can be determined. Such purified inserts can be used for the isolation of full length and genomic sequences, and for the expression of egg surface polypeptide proteins as described below.

5.4 EXPRESSION OF EGG SURFACE POLYPEPTIDE NUCLEIC ACIDS

The nucleotide sequence coding for an egg surface polypeptide protein or a functionally active analog or fragment or other derivative thereof, can be inserted into an appropriate expression vector, *i.e.*, a vector which contains the necessary elements for the transcription and translation of the inserted protein-coding sequence. The necessary transcriptional and translational signals can also be supplied by the native egg surface polypeptide gene and/or its flanking regions. A variety of host-vector systems can be utilized to express the protein-coding sequence. These include but are not limited to mammalian cell systems infected with virus (*e.g.*, vaccinia virus, adenovirus, etc.); insect cell systems infected with virus (*e.g.*, baculovirus); microorganisms such as yeast containing yeast vectors, or bacteria transformed with bacteriophage, DNA, plasmid DNA, or cosmid DNA. The expression elements of vectors vary in their strengths and specificities. Depending on the host-vector system utilized, any one of a number of suitable transcription and translation elements can be used. In yet another embodiment, a fragment of an egg surface polypeptide protein comprising one or more domains of the egg surface polypeptide protein is expressed.

Any of the methods previously described for the insertion of DNA fragments into a vector can be used to construct expression vectors containing a chimeric gene comprising of appropriate transcriptional/translational control signals and the protein coding sequences. These methods can include *in vitro* recombinant DNA and synthetic techniques and *in vivo* recombinants (genetic recombination). Expression of a nucleic acid sequence encoding an egg surface polypeptide protein or peptide fragment can be regulated by a second nucleic acid sequence so that the egg surface polypeptide protein or peptide is expressed in a host transformed with the recombinant DNA molecule. For example, expression of an egg surface polypeptide protein can be controlled by any promoter/enhancer element known in the art. Promoters which can be used to control egg surface polypeptide gene expression include, but are not limited to, the SV40 early promoter region (Benoist and Chambon, 1981, Nature 290:304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto et al., 1980, Cell 22:787-797), the herpes thymidine kinase promoter (Wagner et al., 1981, Proc. Natl. Acad.

Sci. U.S.A. 78:1441-1445), the regulatory sequences of the metallothionein gene (Brinster et al., 1982, Nature 296:39-42); prokaryotic promoters such as the β-lactamase promoter (Villa-Kamaroff et al., 1978, Proc. Natl. Acad. Sci. U.S.A. 75:3727-3731), or the lac promoter (DeBoer et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:21-25); see also "Strategies for Achieving High Level Expression of Genes in *Escherichia coli*" in Microbiological Reviews, 1996, 60:514; plant expression vectors comprising the nopaline synthetase promoter region (Herrera-Estrella et al., Nature 303:209-213) or the cauliflower mosaic virus 35S RNA promoter (Gardner et al., 1981, Nucl. Acids Res. 9:2871), and the promoter of the photosynthetic enzyme ribulose biphosphate carboxylase (Herrera-Estrella et al., 1984, Nature 310:115-120); promoter elements from yeast or other fungi such as the Gal 4 promoter, the ADC (alcohol dehydrogenase) promoter, PGK (phosphoglycerol kinase) promoter, alkaline phosphatase promoter, and the following animal transcriptional control regions, which exhibit tissue specificity and have been utilized in transgenic animals: elastase I gene control region which is active in pancreatic acinar cells (Swift et al., 1984, Cell 38:639-646; Ornitz et al., 1986, Cold Spring Harbor Symp. Quant. Biol. 50:399-409; MacDonald, 1987, Hepatology 7:425-515); a gene control region which is active in pancreatic beta cells (Hanahan, 1985, Nature 315:115-122), an immunoglobulin gene control region which is active in lymphoid cells (Grosschedl et al., 1984, Cell 38:647-658; Adames et al., 1985, Nature 318:533-538; Alexander et al., 1987, Mol. Cell. Biol. 7:1436-1444), mouse mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder et al., 1986, Cell 45:485-495), albumin gene control region which is active in liver (Pinkert et al., 1987, Genes and Devel. 1:268-276), alpha-fetoprotein gene control region which is active in liver (Krumlauf et al., 1985, Mol. Cell. Biol. 5:1639-1648; Hammer et al., 1987, Science 235:53-58; α1-antitrypsin gene control region which is active in the liver (Kelsey et al., 1987, Genes and Devel. 1:161-171), β-globin gene control region which is active in myeloid cells (Mogram et al., 1985, Nature 315:338-340; Kollias et al., 1986, Cell 46:89-94; myelin basic protein gene control region which is active in oligodendrocyte cells in the brain (Readhead et al., 1987, Cell 48:703-712); myosin light chain-2 gene control region which is active in skeletal muscle (Sani, 1985, Nature 314:283-286), and gonadotropic releasing hormone gene control region which is active in the hypothalamus (Mason et al., 1986, Science 234:1372-1378).

Many of the vectors and prokaryotic/eukaryotic host cell systems described supra for constructing gene expression libraries can be used for egg surface polypeptide expression.

In a specific embodiment, a vector is used that comprises a promoter operably linked to an egg surface polypeptide gene nucleic acid, one or more origins of

replication, and, optionally, one or more selectable markers (*e.g.*, an antibiotic resistance gene).

In a specific embodiment, an expression construct is made by subcloning an egg surface polypeptide coding sequence into the EcoRI restriction site of each of the three pGEX vectors (glutathione S-transferase expression vectors; Smith and Johnson, 1988, Gene 7:31-40). This allows for the expression of the egg surface polypeptide protein product from the subclone in the correct reading frame.

Expression vectors containing egg surface polypeptide gene inserts can be identified by three general approaches: (a) nucleic acid hybridization; (b) presence or absence of "marker" gene functions; and (c) expression of inserted sequences. In the first approach, the presence of an egg surface polypeptide gene inserted in an expression vector can be detected by nucleic acid hybridization using probes comprising sequences that are homologous to an inserted egg surface polypeptide gene. In the second approach, the recombinant vector/host system can be identified and selected based upon the presence or absence of certain "marker" gene functions (*e.g.*, thymidine kinase activity, resistance to antibiotics, transformation phenotype, occlusion body formation in baculovirus, etc.) caused by the insertion of an egg surface polypeptide gene in the vector. For example, if the egg surface polypeptide gene is inserted within the marker gene sequence of the vector, recombinants containing the egg surface polypeptide insert can be identified by the absence of the marker gene function. In the third approach, recombinant expression vectors can be identified by assaying the egg surface polypeptide product expressed by the recombinant. Such assays can be based, for example, on the physical or functional properties of the egg surface polypeptide protein *in vitro* assay systems, *e.g.*, binding with anti-egg surface polypeptide protein antibody.

Once a particular recombinant DNA molecule is identified and isolated, several methods known in the art can be used to propagate it. Once a suitable host system and growth conditions are established, recombinant expression vectors can be propagated and prepared in quantity. As previously explained, the expression vectors which can be used include, but are not limited to, the following vectors or their derivatives: human or animal viruses such as vaccinia virus or adenovirus; insect viruses such as baculovirus; yeast vectors; bacteriophage vectors (*e.g.*, lambda phage), and plasmid and cosmid DNA vectors, to name but a few.

In addition, a host cell strain can be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Expression from certain promoters can be elevated in the presence of certain inducers; thus, expression of the genetically engineered egg surface polypeptide protein can

be controlled. Furthermore, different host cells have characteristic and specific mechanisms for the translational and post-translational processing and modification (*e.g.*, glycosylation, phosphorylation of proteins. Appropriate cell lines or host systems can be chosen to ensure the desired modification and processing of the foreign protein expressed. For example, expression in a bacterial system can be used to produce a non-glycosylated core protein product. Expression in yeast will produce a glycosylated product. Expression in mammalian cells can be used to ensure "native" glycosylation of a heterologous protein. Furthermore, different vector/host expression systems can effect processing reactions to different extents.

In other specific embodiments, the egg surface polypeptide, fragment, analog, or derivative can be expressed as a fusion, or chimeric protein product (comprising the protein, fragment, analog, or derivative joined via a peptide bond to a heterologous protein sequence of a different protein). Such a chimeric product can be made by ligating the appropriate nucleic acid sequences encoding the desired amino acid sequences to each other by methods known in the art, in the proper coding frame, and expressing the chimeric product by methods commonly known in the art. Alternatively, such a chimeric product can be made by protein synthetic techniques, *e.g.*, by use of a peptide synthesizer (particularly if some protein sequence is available). The recombinant egg surface polypeptide may not be fully functional for a number of reasons. For example, egg surface polypeptide may be modified *in vivo*. A functional group(s), such as methylation, phosphorylation, or glycosylation, may be added posttranslationally, and play important role(s) in receptor function. Further, egg surface polypeptide may be composed of more than a single polypeptide subunit. In this case recombinant egg surface polypeptide will lack the full activity of the native protein.

5.5 GENERATION OF ANTIBODIES TO EGG SURFACE POLYPEPTIDES

According to the invention, an egg surface protein, its fragments or other derivatives, or analogs thereof, may be used as an immunogen to generate antibodies which immunospecifically bind such an immunogen. Such antibodies include but are not limited to polyclonal, monoclonal, chimeric, single chain, Fab fragments, and an Fab expression library. In a specific embodiment, antibodies to a human egg surface protein are produced. In another embodiment, antibodies to a domain (*e.g.*, the extracelluar domain released by treatment with PI-PLC) of an egg surface protein are produced. In a specific embodiment, fragments of an egg surface protein identified as hydrophilic are used as immunogens for antibody production.

Various procedures known in the art may be used for the production of polyclonal antibodies to an egg surface protein or derivative or analog thereof. In a particular embodiment, rabbit polyclonal antibodies to an epitope of an egg surface protein or fragment, can be obtained. For the production of antibody, various host animals, including but not limited to rabbits, mice, rats, etc can be immunized by injection with the native egg surface proteins, or a synthetic version, or derivative (*e.g.*, fragment) thereof. Various adjuvants may be used to increase the immunological response, depending on the host species, and including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and corynebacterium parvum.

For preparation of monoclonal antibodies directed toward an egg surface protein sequence or analog thereof, any technique which provides for the production of antibody molecules by continuous cell lines in culture may be used. For example, the hybridoma technique originally developed by Kohler and Milstein (1975, Nature 256:495-497), as well as the trioma technique, the human B-cell hybridoma technique (Kozbor et al., 1983, Immunology Today 4:72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., 1985, in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). In an additional embodiment of the invention, monoclonal antibodies can be produced in germ-free animals utilizing recent technology (PCT/US90/02545). According to the invention, human antibodies may be used and can be obtained by using human hybridomas (Cote et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:2026-2030) or by transforming human B cells with EBV virus *in vitro* (Cole et al., 1985, in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, pp. 77-96). In fact, according to the invention, techniques developed for the production of "chimeric antibodies" (Morrison et al., 1984, Proc. Natl. Acad. Sci. U.S.A. 81:6851-6855; Neuberger et al., 1984, Nature 312:604-608; Takeda et al., 1985, Nature 314:452-454) by splicing the genes from a mouse antibody molecule specific for egg surface proteins together with genes from a human antibody molecule of appropriate biological activity can be used; such antibodies are within the scope of this invention.

According to the invention, techniques described for the production of single chain antibodies (U.S. Patent 4,946,778) can be adapted to produce egg surface protein-specific single chain antibodies. An additional embodiment of the invention utilizes the techniques described for the construction of Fab expression libraries (Huse et al., 1989,

Science 246:1275-1281) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity for egg surface proteins, derivatives, or analogs.

Antibody fragments which contain the idiotype of the molecule can be generated by known techniques. For example, such fragments include but are not limited to: the $F(ab')_2$ fragment which can be produced by pepsin digestion of the antibody molecule; the Fab' fragments which can be generated by reducing the disulfide bridges of the $F(ab')_2$ fragment, the Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent, and Fv fragments.

In the production of antibodies, screening for the desired antibody can be accomplished by techniques known in the art, *e.g.* ELISA (enzyme-linked immunosorbent assay). For example, to select antibodies which recognize a specific domain of an egg surface protein, one may assay generated hybridomas for a product which binds to an egg surface protein fragment containing such domain. For selection of an antibody that specifically binds a first egg surface protein homolog but which does not specifically bind a different egg surface protein homolog, one can select on the basis of positive binding to the first egg surface protein homolog and a lack of binding to the second egg surface protein homolog.

Antibodies specific to a domain of an egg surface proteins are also provided.

The foregoing antibodies can be used in methods known in the art relating to the localization and activity of the egg surface proteins of the invention, *e.g.*, for imaging these proteins, measuring levels thereof in appropriate physiological samples, in diagnostic methods, etc.

In another embodiment of the invention (see *infra*), anti-egg surface antibodies and fragments thereof containing the extracellular domain are useful contraceptive vaccines.

5.6 ASSAYS TO IDENTIFY COMPOUNDS THAT MODULATE EGG SURFACE POLYPEPTIDE ACTIVITY

The egg surface proteins of the invention are involved in mediating sperm-egg fusion via a direct interaction between egg surface proteins and a sperm ligand. Thus, the present invention relates to *in vitro* and *in vivo* assay systems, described in the subsections below, which can be used to identify compounds or compositions that modulate the activity of egg surface polypeptide and its interaction with a sperm ligand. Such molecules, such as peptides or non-protein molecules, including organic or inorganic small molecules, large molecules, antibodies, and nucleotide sequences may bind egg surface polypeptide with differing affinities. Such molecules can serve as powerful modulators of fertilization *in vivo*, and can be used therapeutically to modulate the fertility. The screening

assays of the present invention may also be used to identify compounds or compositions that modulate the interaction of egg surface polypeptides with its binding partners, as identified herein.

Methods to screen potential agents for their ability to disrupt or moderate egg surface polypeptide expression and activity can be designed based on the Inventor's discovery of egg surface polypeptides, such as M70, M35/45, H25/40, and GP1-linked ZP3, and their role in egg-sperm fusion and fertilization. The egg surface polypeptide proteins, nucleic acids, and derivatives can be used in screening assays to detect molecules that specifically bind to egg surface polypeptide proteins, derivatives, or nucleic acids, and thus have potential use as agonists or antagonists of egg surface polypeptide, to modulate fertility. In a preferred embodiment, such assays are performed to screen for molecules with potential utility in modulating fertility, thereby useful as contraceptive or sterilization agents. For example, recombinant cells expressing egg surface polypeptide nucleic acids can be used to recombinantly produce an egg surface polypeptide in these assays, to screen for molecules that bind to the egg surface polypeptide. Similar methods can be used to screen for molecules that bind to egg surface polypeptide derivatives or nucleic acids. Methods that can be used to carry out the foregoing are commonly known in the art.

In principle, many methods known to those of skill in the art, can be readily adapted in designing the assays of the present invention. Screening methodologies are well known in the art (see *e.g.*, PCT International Publication No. WO 96/34099, published October 31, 1996, which is incorporated by reference herein in its entirety).

The screening assays, described herein, can be used to identify compounds and compositions including peptides and organic, non-protein molecules that modulate egg surface polypeptide activity. Recombinant, synthetic, and otherwise exogenous compounds may have binding capacity and, therefore, may be candidates for pharmaceutical agents. Alternatively, the proteins and compounds include endogenous cellular components which interact with the identified genes and proteins in vivo. Such endogenous components may provide new targets for pharmaceutical and therapeutic interventions.

Alternatively, any of a variety of exogenous compounds, both naturally occurring and/or synthetic (*e.g.*, libraries of small molecules or peptides), may be screened for binding capacity.

Thus, in a preferred embodiment, both naturally occurring and/or synthetic compounds (*e.g.*, libraries of small molecules or peptides), may be screened for modulating egg surface polypeptide activity. In another series of embodiments, cell lysates or tissue homogenates may be screened for proteins or other compounds which bind to one of the normal or mutant egg surface polypeptide genes and the egg surface polypeptide proteins.

Within the broad category of *in vitro* selection methods, several types of method are likely to be particularly convenient and/or useful for screening test agents. These include, but are not limited to, methods which measure binding of a compound to a egg surface polypeptide, methods which measure a change in the ability of egg surface polypeptide or egg surface polypeptide-positive hybrid cells to interact with an egg surface polypeptide antibody or ligand *in vitro*, and methods which measure the activity or expression of "reporter" protein, that is, an enzyme or other detectable or selectable protein, which has been placed under the control of an egg surface polypeptide gene control region.

The assays of the present invention may be first optimized on a small scale (i.e., in test tubes), and then scaled up for high-throughput assays. The screening assays of the present may be performed *in vitro*, i.e. in test tubes, using purified components or cell lysates. The screening assays of the present invention may also be carried out in intact cells in culture and in animal models. In accordance with the present invention, test compounds which are shown to modulate the activity of egg surface polypeptide *in vitro*, as described herein, will further be assayed *in vivo* in cultured cells and animal models to determine if the test compound has the similar effects *in vivo* and to determine the effects of the test compound on T-cell cytotoxicity, antigen presentation, tumor progression, the accumulation or degradation of positive and negative regulators, cellular proliferation, etc.

In accordance with this embodiment, the systems described below may be formulated into kits. To this end, cells expressing egg surface polypeptide or cell lysates thereof can be packaged in a variety of containers, *e.g.*, vials, tubes, microtiter well plates, bottles, and the like. Other reagents can be included in separate containers and provided with the kit; *e.g.*, positive control samples, negative control samples, buffers, cell culture media, etc.

In one embodiment of the present invention, peptide libraries may be used to screen for agonists or antagonists of egg surface polypeptide. Diversity libraries, such as random or combinatorial peptide or non-peptide libraries can be screened for molecules that specifically bind to egg surface polypeptide. Many libraries are known in the art that can be used, *e.g.*, chemically synthesized libraries, recombinant (*e.g.*, phage display libraries), and *in vitro* translation-based libraries.

Examples of chemically synthesized libraries are described in Fodor et al., 1991, Science 251:767-773; Houghten et al., 1991, Nature 354:84-86; Lam et al., 1991, Nature 354:82-84; Medynski, 1994, Bio/Technology 12:709-710; Gallop et al., 1994, J. Medicinal Chemistry 37(9):1233-1251; Ohlmeyer et al., 1993, Proc. Natl. Acad. Sci. USA 90:10922-10926; Erb et al., 1994, Proc. Natl. Acad. Sci. USA 91:11422-11426; Houghten et al., 1992, Biotechniques 13:412; Jayawickreme et al., 1994, Proc. Natl. Acad. Sci. USA

91:1614-1618; Salmon et al., 1993, Proc. Natl. Acad. Sci. USA 90:11708-11712; PCT Publication No. WO 93/20242; and Brenner and Lerner, 1992, Proc. Natl. Acad. Sci. USA 89:5381-5383.

Examples of phage display libraries are described in Scott and Smith, 1990, Science 249:386-390; Devlin et al., 1990, Science, 249:404-406; Christian, et al., 1992, J. Mol. Biol. 227:711-718; Lenstra, 1992, J. Immunol. Meth. 152:149-157; Kay et al., 1993, Gene 128:59-65; and PCT Publication No. WO 94/18318 dated August 18, 1994.

By way of examples of non-peptide libraries, a benzodiazepine library (*see e.g.*, Bunin et al., 1994, Proc. Natl. Acad. Sci. USA 91:4708-4712) can be adapted for use. Peptoid libraries (Simon et al., 1992, Proc. Natl. Acad. Sci. USA 89:9367-9371) can also be used. Another example of a library that can be used, in which the amide functionalities in peptides have been permethylated to generate a chemically transformed combinatorial library, is described by Ostresh et al. (1994, Proc. Natl. Acad. Sci. USA 91:11138-11142).

Screening the libraries can be accomplished by any of a variety of commonly known methods. See, *e.g.*, the following references, which disclose screening of peptide libraries: Parmley and Smith, 1989, Adv. Exp. Med. Biol. 251:215-218; Scott and Smith, 1990, Science 249:386-390; Fowlkes et al., 1992; BioTechniques 13:422-427; Oldenburg et al., 1992, Proc. Natl. Acad. Sci. USA 89:5393-5397; Yu et al., 1994, Cell 76:933-945; Staudt et al., 1988, Science 241:577-580; Bock et al., 1992, Nature 355:564-566; Tuerk et al., 1992, Proc. Natl. Acad. Sci. USA 89:6988-6992; Ellington et al., 1992, Nature 355:850-852; U.S. Patent No. 5,096,815, U.S. Patent No. 5,223,409, and U.S. Patent No. 5,198,346, all to Ladner et al.; Rebar and Pabo, 1993, Science 263:671-673; and PCT Publication No. WO 94/18318.

In a preferred embodiment, screening can be carried out by contacting the library members with egg surface polypeptide protein (or nucleic acid or derivative) immobilized on a solid phase surface and harvesting those library members that bind to the protein (or nucleic acid or derivative). In a specific embodiment, a library can be screened by passing phage from a continuous phage display library through a column containing purified egg surface polypeptide linked to a solid phase surface, such as plastic beads. By altering the stringency of the washing buffer, it is possible to enrich for phage that express peptides with high affinity for egg surface polypeptide. Phage isolated from the column can be cloned and the affinities of the short peptides can be measured directly. Sequences for more than one oligonucleotide can be combined to test for even higher affinity binding to egg surface polypeptide. Knowing which amino acid sequences confer the strongest binding to egg surface polypeptide, computer models can be used to identify the molecular contacts between egg surface polypeptide and ligand. This will allow the design of non-

protein compounds which mimic those contacts. Such a compound may have the same activity of the peptide and can be used therapeutically, having the advantage of being efficient and less costly to produce.

In another specific embodiment of this aspect of the invention, the solid support is egg surface polypeptide protein (or nucleic acid or derivative) immobilized on a microtiter dish. Cells that express library members are cultivated under conditions that allow expression of the library members in the microtiter dish. Library members that bind to the protein (or nucleic acid or derivative) are harvested. Such methods, are described by way of example in Parmley and Smith, 1988, Gene 73:305-318; Fowlkes et al., 1992, BioTechniques 13:422-427; PCT Publication No. WO 94/18318; and in references cited hereinabove.

In another embodiment of the present invention, interactions between egg surface polypeptide and a test compound may be assayed *in vitro*. Known or unknown molecules are assayed for specific binding to egg surface polypeptide nucleic acids, proteins, or derivatives under conditions conducive to binding, and then molecules that specifically bind to egg surface polypeptide are identified. The two components can be measured in a variety of ways. One approach is to label one of the components with an easily detectable label, place it together with a test component(s) under conditions that allow binding to occur, perform a separation step which separates bound labeled component from unbound labeled component, and then measure the amount of bound component. In one embodiment, egg surface polypeptide can be labeled and added to a test agent, using conditions that allow binding to occur. Binding of the test agent can be determined using polyacrylamide gel analysis to compare complexes formed in the presence and absence of the test agent.

In another embodiment of the present invention, the screening may be performed by adding the labeled egg surface polypeptide to *in vitro* translation systems such as a rabbit reticulocyte lysate (RRL) system and then proceeding with the binding reaction. *In vitro* translation-based libraries include but are not limited to those described in PCT Publication No. WO 91/05058 dated April 18, 1991; and Mattheakis et al., 1994, Proc. Natl. Acad. Sci. USA 91:9022-9026.

In another embodiment, binding of egg surface polypeptide to a test agent may be assayed in intact cells in culture or in animal models in the presence and absence of the test compound. For example, a labeled test agent may be mixed with eggs in culture, or to crude extracts obtained from animal tissue samples, and the test compound may be added. Binding can be assayed using microscopy or confocal microscopy, for example. In yet another embodiment, the test agent may be assayed in intact cells in animal models. A

labeled test agent may be administered directly to an animal. The uptake of the test agent may be measured. For these assays, host cells to which the test compound is added may be genetically engineered to express egg surface polypeptide and its target interactor (such as an egg surface polypeptide antibody or an egg surface polypeptide ligand) which may be transient, induced or constitutive, or stable. For the purposes of the screening methods of the present invention, a wide variety of host cells may be used including, but not limited to, tissue culture cells, mammalian cells, yeast cells, and bacteria. Each cell type has its own set of advantages and drawbacks. Mammalian cells, such as egg cells which express the egg surface polypeptides of the invention, may be a preferred cell type in which to carry out the assays of the present invention. Bacteria and yeast are relatively easy to cultivate but process proteins differently from mammalian cells.

In a specific embodiment, the invention provides for a method for detecting the interaction between egg surface polypeptide and a known potential ligand, such as a sperm integrin. Insect cells can be infected with baculoviruses co-expressing egg surface polypeptide and the known sperm integrin, and cell extracts can be prepared and analyzed for protein-protein interactions. Protein-protein interactions can be analyzed by methods known in the art, such as Western blotting or immune precipitation using egg surface polypeptide specific antibodies together with an anti-integrin antibody, and analyzing complexes by polyacrylamide gel electrophoresis.

The invention further provides methods for screening cells having egg surface polypeptide proteins (or fragments thereof) as one of their cell surface membrane components for known cell surface molecules as potential ligands. For example, cells engineered to express egg surface polypeptide nucleic acids can be used to recombinantly produce egg surface polypeptide proteins either wild-type or dominant negative mutants in cells that also express a putative egg surface polypeptide binding partner molecule. Potential candidates for an egg surface polypeptide binding partner include, but are not limited to, such known substrates as sperm integrin. Extracts can also be used to test whether the presence of egg surface polypeptide increases or decreases the level of the potential binding partner.

In another embodiment, the two-hybrid system for selecting interacting proteins or peptides in yeast (Fields and Song, 1989, Nature 340:245-246; Chien et al., 1991, Proc. Natl. Acad. Sci. USA 88:9578-9582) can be used to identify molecules that specifically bind to egg surface polypeptide protein or derivative.

In yet another embodiment of the present invention, peptide libraries may be used to identify for unknown potential ligands of egg surface polypeptide. Diversity libraries, such as random or combinatorial peptide libraries can be screened for molecules

that specifically bind to egg surface polypeptide. Many libraries are known in the art that can be used, *e.g.*, chemically synthesized libraries, recombinant (*e.g.*, phage display libraries), and *in vitro* translation-based libraries.

Once a substrate or interacting protein is identified, then one can assay for modulators of the egg surface polypeptide interaction with such a protein. The present invention provides for methods of detecting agonists and antagonists of such interactions.

Recombinant egg surface polypeptide and putative agonist or antagonist molecules can be incubated together, under conditions that allow binding to occur, such as 37°C for 30 minutes. Protein-protein complex formation can be detected by acrylamide gel analysis, by methods known in the art. This assay can be used to identify modulators of interactions of sperm and egg surface proteins involved in fertilization.

Purified or partially purified components which have been determined to interact with one another by the methods described above can be placed under conditions in which the interaction between them would normally occur, with and without the addition of the test agent, and the procedures previously established to analyze the interaction can be used to assess the impact of the test agent. In this approach, the purified or partially purified components may be prepared by fractionation of extracts of eggs, or they may be obtained by expression of cloned genes or cDNAs or fragments thereof, optionally followed by purification of the expressed material.

5.7 VACCINES AGAINST EGG SURFACE PROTEINS

Mouse or hamster egg surface proteins can be used in any of a variety of vaccines in human and non-human animals. The present invention encompasses vaccines useful for contraception. In one aspect of the invention, egg surface polypeptides involved in sperm-egg fusion, such as M70, M35/45, H25/40, and GP1-linked ZP3, are delivered to a subject to elicit an active immune response. The vaccine acts as a temporary and reversible antagonist of the function of the egg surface proteins of the invention. For example, such vaccines could be used for active immunization of a subject, to raise an antibody response to temporarily block the sperm's access to the egg plasma antigen. In one aspect of the invention, an antigen could be administered at a certain period of the month, for example during ovulation of a female subject to block fertilization.

In another aspect of the invention, egg surface polypeptides involved in sperm-egg fusion, such as GPI-linked ZP3, M70, M35/45, H25/40, and GP1-linked ZP3, are useful as vaccines for permanent sterilization of a subject. Such vaccines can be used to elicit a T-cell mediated attack on the eggs, having an othoritic effect, useful as a method for

irreversible sterilization. Methods for generating T-cell specific responses, such as adoptive immunotherapy, are well known in the art (see, for example, Vaccine Design, Michael F. Powell and Mark J. Newman Eds., Plenum Press, New York, 1995, pp 847-867). Such techniques may be particular useful for vetinary contraceptive or sterilization purposes, where a single dose vaccination may be desirable.

5.7.1 Vaccine Formulations and Methods of Administration

Egg surface protein antigens can be produced in large amounts and purified for use in vaccine preparations. The egg surface proteins of the invention also have utility in immunoassays, e.g., to detect or measure in a sample of body fluid from a vaccinated subject the presence of antibodies to the antigen, and thus to diagnose and/or to monitor immune response of the subject subsequent to vaccination.

The preparation of vaccines containing an immunogenic polypeptide as the active ingredient is known to one skilled in the art (see, for example, Vaccine Design, Michael F. Powell and Mark J. Newman Eds., Plenum Press, New York, 1995, pp 821-902)

5.7.1.1 Determination of Vaccine Efficacy

The immunopotency of egg surface protein antigens can be determined by monitoring the immune response in test animals following immunization with the egg surface protein antigen, or by use of any immunoassay known in the art. Generation of a humoral (antibody) response and/or cell-mediated immunity, may be taken as an indication of an immune response. Test animals may include mice, hamsters, dogs, cats, monkeys, rabbits, chimpanzees, etc., and eventually human subjects.

Methods of introducing the vaccine may include oral, intravaginal, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, and via scarification (scratching through the top layers of skin, *e.g.*, using a bifurcated needle) or any other standard routes of immunization. The immune response of the test subjects can be analyzed by various approaches such as: the reactivity of the resultant immune serum to the egg protein antigen, as assayed by known techniques, e.g., immunosorbent assay (ELISA), immunoblots, radioimmunoprecipitations, etc., or in the case where the egg protein antigen displays antigenicity or immunogenicity, by protection of the immunized host against fertilization in the immunized host.

As one example of suitable animal testing of an egg surface protein vaccine, the vaccine of the invention may be tested in rabbits for the ability to induce an antibody response to the egg surface protein antigen. Male specific-pathogen-free (SPF) young adult New Zealand White rabbits may be used. The test group each receives a fixed

concentration of the vaccine. A control group receives an injection of 1 mM Tris-HCl pH 9.0 without the egg surface protein antigen.

Blood samples may be drawn from the rabbits every one or two weeks, and serum analyzed for antibodies to the egg surface protein. The presence of antibodies specific for the antigen may be assayed, e.g., using an ELISA.

5.7.1.2 Vaccine Formulations

Suitable preparations of such vaccines include injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, suspension in, liquid prior to injection, may also be prepared. The preparation may also be emulsified, or the polypeptides encapsulated in liposomes. The active immunogenic ingredients are often mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water saline, dextrose, glycerol, ethanol, or the like and combinations thereof. In addition, if desired, the vaccine preparation may also include minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, and/or adjuvants which enhance the effectiveness of the vaccine.

Examples of adjuvants which may be effective, include, but are not limited to: aluminum hydroxide, N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acetyl-nor-muramyl-L-alanyl-D-isoglutamine, N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-2'-dipalmitoyl-sn-glycero-3-hydroxyphosphoryloxy)-ethylamine.

The effectiveness of an adjuvant may be determined by measuring the induction of antibodies directed against an immunogenic polypeptide containing an egg surface protein polypeptide epitope, the antibodies resulting from administration of this polypeptide in vaccines which are also comprised of the various adjuvants.

The polypeptides may be formulated into the vaccine as neutral or salt forms. Pharmaceutically acceptable salts include the acid addition salts (formed with free amino groups of the peptide) and which are formed with inorganic acids, such as, for example, hydrochloric or phosphoric acids, or organic acids such as acetic, oxalic, tartaric, maleic, and the like. Salts formed with free carboxyl groups may also be derived from inorganic bases, such as, for example, sodium potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine and the like.

The vaccines of the invention may be multivalent or univalent. Multivalent vaccines are made from recombinant viruses that direct the expression of more than one antigen.

The patient to which the vaccine is administered is preferably a mammal, most preferably a human, but can also be a non-human animal including but not limited to cows, horses, sheep, pigs, fowl (*e.g.*, chickens), goats, cats, dogs, hamsters, mice and rats.

The vaccine formulations of the invention comprise an effective immunizing amount of the egg surface protein and a pharmaceutically acceptable carrier or excipient. Vaccine preparations comprise an effective immunizing amount of one or more antigens and a pharmaceutically acceptable carrier or excipient. Pharmaceutically acceptable carriers are well known in the art and include but are not limited to saline, buffered saline, dextrose, water, glycerol, sterile isotonic aqueous buffer, and combinations thereof. One example of such an acceptable carrier is a physiologically balanced culture medium containing one or more stabilizing agents such as stabilized, hydrolyzed proteins, lactose, etc. The carrier is preferably sterile. The formulation should suit the mode of administration.

The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. The composition can be a liquid solution, suspension, emulsion, tablet, pill, capsule, sustained release formulation, or powder. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, etc.

Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water free concentrate in a hermetically sealed container such as an ampoule or sachette indicating the quantity of active agent. Where the composition is administered by injection, an ampoule of sterile diluent can be provided so that the ingredients may be mixed prior to administration.

In a specific embodiment, a lyophilized egg surface protein polypeptide of the invention is provided in a first container; a second container comprises diluent comprising an aqueous solution of 50% glycerin, 0.25% phenol, and an antiseptic (*e.g.*, 0.005% brilliant green).

The precise dose of vaccine preparation to be employed in the formulation will also depend on the route of administration, and the nature of the patient, and should be decided according to the judgment of the practitioner and each patient's circumstances according to standard clinical techniques. An effective immunizing amount is that amount sufficient to produce an immune response to the antigen in the host to which the vaccine preparation is administered.

Use of purified antigens as vaccine preparations can be carried out by standard methods. For example, the purified protein(s) should be adjusted to an appropriate concentration, formulated with any suitable vaccine adjuvant and packaged for use.

Suitable adjuvants may include, but are not limited to: mineral gels, *e.g.*, aluminum hydroxide; surface active substances such as lysolecithin, pluronic polyols; polyanions; peptides; oil emulsions; alum, and MDP. The immunogen may also be incorporated into liposomes, or conjugated to polysaccharides and/or other polymers for use in a vaccine formulation. In instances where the recombinant antigen is a hapten, *i.e.*, a molecule that is antigenic in that it can react selectively with cognate antibodies, but not immunogenic in that it cannot elicit an immune response, the hapten may be covalently bound to a carrier or immunogenic molecule; for instance, a large protein such as serum albumin will confer immunogenicity to the hapten coupled to it. The hapten-carrier may be formulated for use as a vaccine.

Effective doses (immunizing amounts) of the vaccines of the invention may also be extrapolated from dose-response curves derived from animal model test systems.

The invention also provides a pharmaceutical pack or kit comprising one or more containers comprising one or more of the ingredients of the vaccine formulations of the invention. Associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

The present invention thus provides a method of immunizing an animal, comprising administering to the animal an effective immunizing dose of a vaccine of the present invention.

5.7.1.3 Packaging

The compositions may, if desired, be presented in a pack or dispenser device which may contain one or more unit dosage forms containing the active ingredient. The pack may for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration. Compositions comprising a compound of the invention formulated in a compatible pharmaceutical carrier may also be prepared, placed in an appropriate container, and labelled for treatment of an indicated condition.

5.7.2 Use of Antibodies Generated by Vaccines of the Invention

The antibodies generated against the antigen by immunization with the egg surface proteins of the present invention also have potential uses in vaccination against fertilization, sterilization, diagnostic immunoassays, passive immunotherapy, and generation of antiidiotypic antibodies.

The generated antibodies may be isolated by standard techniques known in the art (*e.g.*, immunoaffinity chromatography, centrifugation, precipitation, etc.) and used in diagnostic immunoassays. The antibodies may also be used to monitor treatment and/or disease progression. Any immunoassay system known in the art, such as those listed *supra*, may be used for this purpose including but not limited to competitive and noncompetitive assay systems using techniques such as radioimmunoassays, ELISA (enzyme-linked immunosorbent assays), "sandwich" immunoassays, precipitin reactions, gel diffusion precipitin reactions, immunodiffusion assays, agglutination assays, complement-fixation assays, immunoradiometric assays, fluorescent immunoassays, protein A immunoassays and immunoelectrophoresis assays, to name but a few.

The vaccine formulations of the present invention can also be used to produce antibodies for use in passive immunotherapy, in which short-term protection of a host is achieved by the administration of pre-formed antibody directed against a heterologous organism.

The antibodies generated by the vaccine formulations of the present invention can also be used in the production of antiidiotypic antibody. The antiidiotypic antibody can then in turn be used for immunization, in order to produce a subpopulation of antibodies that bind the initial antigen of the pathogenic microorganism (Jerne, 1974, Ann. Immunol. (Paris) 125c:373; Jerne, et al., 1982, EMBO J. 1:234).

In immunization procedures, the amount of immunogen to be used and the immunization schedule will be determined by a physician skilled in the art and will be administered by reference to the immune response and antibody titers of the subject.

6. EXAMPLE: 70kDa and 35-45 kDa Egg Surface Protein Clusters Involved in Sperm-Oolemma Binding and Fusion

The experiments presented in the following Example demonstrate the successful identification and characterization of glycosylated phosphatidylinositol (GPI)-anchored egg surface proteins required for fertilization. The results presented herein show that glycosyl-phosphatidylinositol (GPI)-anchored egg surface proteins are required for sperm to bind to and fuse with the egg. Thus, while treatment of mouse sperm with PI-PLC had no significant effect on either sperm-zona pellucida binding or sperm-egg binding and fusion, treatment of zona-intact or zona-free oocytes with PI-PLC blocked fertilization. The GPI-anchored egg surface proteins were characterized by 2-dimensional avidin blotting. As demonstrated herein, biotinylated mouse oocytes released protein clusters of approximately 70 kDa (pI 5) and 35-45 kDa (pI 5.5) following PI-PLC treatment.

6.1 INTRODUCTION

The role of GPI-anchored proteins in gamete interaction has yet to be thoroughly investigated. Mouse sperm surface hyaluronidase (also known as PH-20) is GPI-anchored and is thought to aid sperm in passage through the cumulus oopherous and possibly the zona pellucida by hydrolyzing the extracellular matrix protein, hyaluronic acid (Gmachl and Kreil, 1993, Proc. Nat. Acad. Sci. USA 90: 3569-3573; Myles and Primakoff, 1997, Biol. Reprod., 56: 320-327). Sperm agglutination antigen-1 (SAGA-1) is another sperm surface protein which has been shown to be a GPI-linked. While its role in fertilization has yet to be elucidated, in vitro assays have demonstrated that anti-SAGA-1 monoclonal antibodies agglutinate human sperm (Diekman et al., 1997, Biol. Reprod. 57: 1136-1144).

The effect of phosphatidylinositol-specific phospholipase C (PI-PLC) on mouse sperm-egg interaction was investigated in this study to determine if GPI-anchored proteins are involved in mammalian fertilization. In initial studies, mouse fertilization was blocked when sperm and zona-intact eggs were pre-treated for 30 min with a highly purified preparation of recombinant PI-PLC prior to gamete co-incubation. The stage in the fertilization cascade at which PI-PLC exerted its inhibitory effect, and whether one or both gametes were affected by PI-PLC was then investigated. Upon finding that PI-PLC treatment of zona-free oocytes, but not sperm, blocked sperm-egg binding and fusion, the released oolemmal GPI-anchored protein(s) were characterized using two-dimensional (2D) gel electrophoresis and cell surface labeling.

6.2 MATERIALS AND METHODS

PI-PLC Preparation

Recombinant PI-PLC was isolated from cultured supernatants of *Bacillus subtilis* (BG2320) transfected with the PI-PLC gene from *B. thuringiensis* (Henner et al., 1988, Nucl. Acid. Res. 16: 10383-10383). For details on PI-PLC purification see Low et al. (1988, J. Imm. Meth. 113: 101-111).

Gamete preparation

Preparation of mouse gametes for IVF was carried out essentially as described by (Bleil, 1991, Meth. Enz. 225: 253-263). Incubation of mouse gametes was performed in microdrops under paraffin oil at 37^{0}C and 5% CO_2 in Medium 199 (M199, Gibco, Grand Island, NY) supplemented with 4 mg/ml bovine serum albumin (BSA, Sigma, St. Louis, MO) and 3.5 mM sodium pyruvate (Gibco). Epididymides were collected from

sacrificed ICR strain retired breeders, placed in 250 µl medium, minced with watchmaker forceps, and sperm were allowed to swim out from the epididymides for 15 min. The sperm suspension was then placed under 1.5 ml medium and sperm were allowed to swim up for at least 1 h. Cumulus-oocyte complexes were collected from superovulated ICR strain females in M199. Cumulus cells were removed by treating eggs for 3 min with 1 mg/ml hyaluronidase (Sigma) in M199 and the eggs were then washed in six consecutive 50µl drops of M199. Following cumulus cell removal, oocytes were washed by passing the eggs through 50 µl drops of media covered with mineral oil using a pulled, heat polished, Pasteur pipette (employed in all experiments).

Sperm-zona binding and fertilization of zona-intact oocytes following pre-treatment of sperm and eggs with PI-PLC

Swim up sperm (approximately $3x10^6$ sperm/ml) were treated for 30 min with either 1U/ml PI-PLC or 1U/ml of heat inactivated (95^0C for 5 min) PI-PLC in 100 µl of M199 under oil. Zona-intact oocytes (approximately 30 eggs per group) were treated for 30 min with either 1U/ml PI-PLC or 1U/ml inactivated PI-PLC in 100 µl of M199 under oil. Treated sperm were then added to the incubation drops containing treated eggs with a final droplet volume of 100 µl and a final sperm concentration of $1x10^6$ sperm/ml. Thus, PI-PLC was present in the incubation droplet during gamete interaction.

For the sperm-zona binding assay, the gametes were co-incubated for 1 h, washed gently 3 times in M199, and fixed in PBS containing 4% paraformaldehyde for 1 h. To quantitate binding, the oocytes were placed in a phosphate buffered saline (PBS)/4% paraformaldehyde solution between a microscope slide and an elevated cover slip. The oocytes were visualized at 200X using a light microscope (Zeiss Axioplan) and a single focal plane for each oocyte was selected in which the widest diameter of the zona pellucida could be visualized. The number of bound sperm in that focal plane was then determined.

To study fertilization of zona-intact oocytes, sperm were added to oocytes, the gametes were co-cultured for 2 h, washed 3 times in M199 to remove the PI-PLC and supernumerary sperm and incubated overnight at 37°C and 5% CO_2. To visualize sperm located within the perivitelline space following overnight incubation, gametes were incubated in 1 µM Hoechst dye #33342 (Sigma) for 10 min and washed 6 times in M199. The zygotes were then viewed at 200X as described above and zygotes with two blastomeres were scored as fertilized while one-celled oocytes were scored as unfertilized.

Fertilization of zona-intact oocytes which had been pre-treated with PI-PLC and washed prior to incubation with untreated sperm

Zona-intact oocytes (approximately 20 eggs per group) were treated for 30 min with either 1U/ml PI-PLC or 1U/ml inactivated PI-PLC in 100 µl of M199 under oil. The oocytes were then washed through six drops of M199. Untreated sperm were then added to the incubation drops containing treated eggs at a final volume of 100 µl and sperm concentration of $1X10^6$ sperm/ml. The gametes were then co-cultured for 2 h, washed 3 times in M199 and incubated overnight at 37°C and 5% CO_2. Zygotes with two blastomeres were scored as fertilized while one-celled oocytes were scored as unfertilized.

Sperm-oolemma binding and fusion following pre-treatment of either sperm or zona-free eggs with PI-PLC

Zonae pellucida were loosened by treating eggs with M199 containing 10 µg/ml chymotrypsin (Sigma) for 1 min. The eggs were then washed 6 times in M199 and loosened zonae were removed by mechanical agitation using a pulled Pasteur pipette. The eggs were allowed to recover from chymotrypsin treatment for 4 h in M199. The oocytes (approximately 15 oocytes per group) were then pre-loaded with 1 µM Hoechst dye #33342 (Sigma) for 10 min and washed 6 times in M199. Swim up sperm were collected and prepared as described in the gamete preparation section and allowed to capacitate for four hours during the oocyte recovery period. To evaluate the effect of treating only sperm with PI-PLC on subsequent sperm-egg binding and fusion, swim up sperm (approximately $3X10^6$ sperm/ml) were treated for 30 min with either 1U/ml PI-PLC or 1U/ml of heat inactivated PI-PLC in 100 µl of M199 under oil. The sperm were then washed 2 times by centrifugation (five min at 1000 g) in five ml volumes of M199 in 15 ml centrifuge tubes to remove the PI-PLC. The treated sperm were then added to untreated eggs (approximately 15 eggs per group) at a concentration of $1x10^5$ sperm/ml and the gametes were co-incubated for 40 min in 20 µl drops followed by gentle washing.

To evaluate the effect of treating only oocytes with PI-PLC on subsequent sperm-egg binding and fusion, the eggs were treated with either 1U/ml PI-PLC or heat inactivated PI-PLC for 30 min in 50 µl drops of M199 and washed through six 50 µl drops of M199 to remove the PI-PLC. The treated eggs were then incubated with untreated sperm ($1x10^5$ sperm/ml) for 40 min in 20 µl drops containing approximately fifteen oocytes per group followed by gentle washing of the eggs in M199. To determine if the effect of PI-PLC on zona-free oocytes was dose-dependent, oocytes were treated with either 5U/ml heat inactivated PI-PLC or increasing concentrations of PI-PLC (0-5U), washed, and incubated with untreated sperm as described above. To evaluate sperm-egg binding and fusion, the eggs from each group were then placed in M199 between a microscope slide and an elevated cover slip and visualized at 200X. Binding to the egg was scored by counting the total number of sperm bound per oocyte using phase contrast. Fusion with the egg was

scored by counting the number of decondensed sperm heads within each oocyte using fluorescent microscopy.

Artificial activation of oocytes following PI-PLC treatment

In order to ensure that PI-PLC treated eggs remained viable following PI-PLC treatment, zona-free eggs were treated with either 1U/ml PI-PLC or 1U/ml heat inactivated PI-PLC for 30 min as described in the sperm-oolemma binding and fusion assay. Immediately prior to egg activation, a small sample of oocytes were visualized using fluorescent microscopy to ensure that the main pool of oocytes were in metaphase II arrest following PI-PLC treatment. The remaining oocytes were activated by placing the eggs in 0.5 μM calcium ionophore (A23187, Sigma) for 5 min followed by three washes. The eggs were incubated for 40 min and oocytes were observed as described above. The eggs were considered activated if they had advanced from metaphase II arrest to anaphase II or telophase II (with second polar body).

Binding of beads coated with anti-integrin antibodies to eggs following PI-PLC treatment

Two-tenths μm yellow-green sulfate-derivatized latex beads (Molecular Probes, Eugene, OR) were coated with the goat (Go) H3 mAb (IgG2a,) to the α6 integrin subunit or an irrelevant control mAb (anti-MLV) of the same isotype as follows: beads from 10 μl of a 2% bead suspension were incubated with 10 μl of antibody (0.4 mg/ml) for 3 h at 4°C on an orbital platform mixer (Clay Adams, Parsippany, NJ). Beads were then washed twice with PBS, quenched for 1 h with 0.2 mg/ml goat anti-rabbit IgG (Sigma), washed twice with PBS, and then resuspended to 0.2% in PBS. Beads were used on the day of preparation and were sonicated 3 times for 5 sec each at 4° C immediately prior to use. Next, 20-40 zona-free eggs were either sham treated or treated with 1U/ml PI-PLC for 30 min, washed, and the protein-coated fluorescent beads (final concentration 0.02%) were added to each group. The eggs were incubated in a 5% CO_2 incubator and gently agitated every 15 min. After 1 h at 37°C, the eggs were washed through three 100 μl drops of fresh medium using an ~100 μm glass pipette. Following washes the eggs were placed 24-well dishes in small drops and overlaid with light mineral oil for imaging by confocal microscopy.

Two-Dimensional Gel Electrophoresis

Cumulus-oocyte complexes were collected and cumulus cells were removed as described above. To facilitate the collection of large numbers of zona-free oocytes for optimization of the 2D gel electrophoresis experiments, acid Tyrodes was used to removed

the zona pellucidae instead of chymotrypsin. To ensure that the acid Tyrodes method of zona removal did not affect the experimental outcome, the experiment was also performed using oocytes which had their zona removed using the chymotrypsin treatment method described in the sperm-oolemma binding and fusion section and the results were compared. For removal of zonae using acid Tyrodes, oocytes were placed in acid Tyrodes for 15 sec followed immediately by four washes in M199 (Evans et al., 1997, Dev. Biol. 187:79-93). The eggs were then allowed to recover for 4 h at 37°C and 5% CO_2. The eggs were then washed six times in BWW media (Irvine Scientific, Santa Ana, CA) containing 100 µg/ml polyvinylalcohol (PVA, Sigma), biotinylated with 2 µg/ml Sulfo-NHS biotin (Pierce, Rockford, IL) in BWW/PVA for 7 min at room temperature, and washed six times in BWW/PVA. The eggs were then split into two groups of 100 and either mock treated or treated with 1U/ml PI-PLC in 20 µl drops for 30 min. The supernatants were removed, the eggs were washed six times, and the oocytes and the oocyte supernatants were then frozen at -70°C in BWW/PVA containing protease inhibitors (Complete™, Boehringer Mannheim, Mannheim, Germany). The oocytes and supernatants were extracted in Celis lysis buffer containing 2% (v:v) NP-40, 9.8M urea, 100mM dithiothreitol (DTT), 2% ampholines (pH 3.5-10), and protease inhibitors for 30 min at room temperature (Rasmussen et al., 1991, Electrophoresis 12: 873-882). Isoelectric focusing (IEF) was performed using the Mini-PROTEAN II tube cell (Bio RAD, Richmond, CA) apparatus and protocol with an ampholine mixture (Pharmacia Biotech, Uppsala, Sweden) of pH 3.5-5 (30%), 3.5-10 (40%), 5-7 (20%), and 7-9 (10%). The tube gels were placed on 12 % mini slab gels and the focused proteins were separated in the second dimension at 20 mA per gel. The proteins were then electroblotted to nitrocellulose membranes at 125 mA for 45 min. The nitrocellulose membranes were blocked in PBS with 0.1% Tween and 5% dried milk for 30 min at room temperature, washed 1 time in PBS/0.1% Tween, and probed with 20 µg/ml strepavidin-HRP (Pierce) for 30 min at room temperature. The blots were washed 3 times 15 min in PBS/0.1% Tween (10 min per wash) and developed using enhanced chemiluminescence (Amersham Corp, Buckinghamshire, U.K.) for 5 min. As a control for determining the charge, mass, and location of the PI-PLC enzyme, one unit (1µg) of the PI-PLC preparation was mixed with Celis buffer and separated on a two-dimensional electrophoretic gel as described above and silver stained according to (Hochstrasser et al., 1988, Anal. Biochem. 173: 424-435).

Statistical Analysis

All in vitro assays were repeated at least three times. Experimental and control group averages were reported as means +/- the standard deviation. Groups were

compared using the students T test and differences were reported at the 0.05 level of significance.

6.3 RESULTS

In Vitro Assays

Co-culture of mouse sperm and zona-intact eggs in the presence of PI-PLC blocked fertilization

To determine the effects of PI-PLC on sperm-zona pellucida binding, mouse sperm and eggs were pre-treated separately with either 1U/ml heat inactivated PI-PLC or 1U/ml PI-PLC for 30 min, and co-cultured in the presence of PI-PLC. Following one hour of co-culture, sperm were observed to bind abundantly to the zona of both control (Fig. 1A) and treated gametes (Fig. 1B). Quantitation revealed no significant difference in the number of sperm-bound per zona pellucida between the control group (43.9 sperm/zona) and the PI-PLC treated group (45.9 sperm/zona) (Fig. 1E).

To determine the effects of PI-PLC on fertilization of zona-intact oocytes, sperm and eggs were pre-treated separately with PI-PLC as described above, co-cultured in the presence of PI-PLC for two hours to permit fertilization, washed 3 times to remove the PI-PLC and supernumerary sperm, and incubated overnight. It is noteworthy that the molecular weight of PI-PLC is 30-34 kDa and the zona pellucida is freely permeable to molecules having a mass of less than 170 kDa (Legge, 1995, J. Exp. Zool. 271: 145-150); therefore the oolemma was likely exposed to PI-PLC during treatment. Twenty four hours following gamete co-culture, the majority of eggs in the control group had undergone cleavage whereas few oocytes had done so in the PI-PLC treated group (Fig. 1C and 1D). Quantitation revealed a significant reduction in the fertilization rate from 59.6% in the inactivated PI-PLC control to 2.8% in the PI-PLC treated group (Fig. 1E). A most striking observation was the large number of sperm which accumulated within the perivitelline space of uncleaved PI-PLC treated eggs (Fig. 1D, inset) when compared to controls (Fig. 1C, inset).

PI-PLC effect on fertilization of zona-intact eggs

The fertilization experiments utilizing zona-intact oocytes demonstrated that pre-treatment of sperm and eggs with PI-PLC followed by gamete co-incubation in the presence of PI-PLC inhibited fertilization while sperm-zona binding was unaffected. It then became of interest to determine whether the fertilization inhibition of zona-intact eggs by PI-PLC was due to an effect on the sperm or the egg. Therefore, either sperm or eggs which

had been treated with PI-PLC and washed free of enzyme prior to gamete co-incubation were used to attempt to fertilize zona-intact oocytes. When zona-intact oocytes were treated with 1U/ml PI-PLC for 30 min and washed prior to incubation with untreated sperm, fertilization rates were reduced from 63% in the control group to 3% in the PI-PLC treatment group. This reduction in fertilization is similar to that observed when PI-PLC was present in the fertilization medium during gamete interaction. This result suggested that PI-PLC blocked fertilization by affecting the oolemma.

In evaluating the effect of PI-PLC on sperm, both the control and treatment groups underwent a loss in motility following centrifugal washing and neither were able to fertilize zona-intact eggs. Therefore, it was not possible to establish if PI-PLC treatment affected sperm fertilizing ability using zona-intact eggs. The reduction in motility, however, did not affect sperm-oolemma binding and fusion, and the locus of PI-PLC action was refined using zona-free eggs.

Pre-treatment of zona-free oocytes with PI-PLC inhibits both sperm-egg binding and fusion

When sperm were treated with either 1U/ml heat inactivated PI-PLC or 1U/ml PI-PLC for 30 min, washed, and incubated with untreated zona-free oocytes, no statistical difference was observed in sperm-egg binding or fusion between the control (Fig. 2A) and treatment group (Fig. 2B). In the control group 5.4 sperm bound per egg whereas 8.0 sperm bound per egg in the PI-PLC treated group (Fig. 2E). Similarly, when sperm were treated with PI-PLC, there was no significant difference in sperm-egg fusion when comparing the control group (1.7 sperm fused per egg) to the treatment group (1.5 sperm fused per egg). By contrast, when zona-free oocytes were treated with either 1U/ml heat inactivated PI-PLC or 1U/ml PI-PLC for 30 min, washed, and incubated with untreated sperm, a significant decrease in binding and fusion was observed in the treatment group (Fig. 2D) when compared to controls (Fig. 2C). In the control group 6.2 sperm bound per egg and 1.9 sperm fused per egg compared to the PI-PLC treated group where 2.1 sperm bound per egg and 0.02 sperm fused per egg (Fig. 2E). These results further support our results that the PI-PLC effect on fertilization is mediated at the oolemma. Of interest was the observation that while most of the PI-PLC treated oocytes had only 0 to 2 bound sperm, a small percent of the treated eggs were bound by numerous sperm yet fusion was still blocked (see oocyte indicated by arrow in Fig. 2D).

Treatment of zona-free oocytes with PI-PLC inhibits sperm-egg binding and fusion in a dose-dependent manner

Oocytes were treated with either 5U/ml of heat inactivated PI-PLC, no PI-PLC, or increasing amounts of PI-PLC (0, 0.05, 0.1, 0.5, 1, 5U/ml), washed, and incubated with untreated sperm to determine if the inhibitory effect of PI-PLC on the oolemma was dose-dependent. Results showed that, as the concentration of PI-PLC was increased, sperm-egg binding and fusion rates decreased in a dose-dependent manner (Fig. 3). The maximal inhibitory effect on sperm-egg binding was reached at 5U/ml while the maximal inhibitory effect on fusion occurred at 1U/ml. The recommended dose for releasing most GPI-anchored proteins from intact cells using *B. thuringiensis*-derived PI-PLC is 1U/ml. The dose-dependent inhibition of both sperm-egg binding and fusion by PI-PLC supports the hypothesis that treating oocytes with PI-PLC releases GPI-anchored proteins from the oolemma which are required for fertilization.

Purity of the recombinant PI-PLC enzyme

To evaluate the purity of the PI-PLC preparation, an aliquot of the enzyme was separated by 2D electrophoresis and the gel was silver stained. Results showed that only one prominent protein spot (approximately MW 30 kDa, approximate pI 6) and several smaller protein spots immediately surrounding the prominent protein (possibly isoforms of PI-PLC) could be visualized (Fig. 4). The reported molecular weight of PI-PLC under reducing conditions is 30-35 kDa (Low and Saltiel, 1988, Science, 239: 268-275). These results indicate that the recombinant PI-PLC preparation used for the experiments in this study was highly purified.

Treatment of zona-free eggs with PI-PLC had no observable effect on artificial egg activation

Artificial activation of oocytes was performed to ensure that the oocytes remained viable following PI-PLC treatment. Zona-free eggs were either treated with 1U/ml heat inactivated PI-PLC or 1U/ml PI-PLC, washed, and samples of control and treated eggs were observed to ensure that the oocytes remained in meiotic arrest following PI-PLC treatment. All oocytes (14 oocytes per group) which were observed in both control (Fig 5A, inset) and treatment groups (Fig 5B, inset) remained in meiotic arrest. The remaining oocytes were then artificially activated with 0.5 μm calcium ionophore A23187 for 5 min, washed, and cultured for 40 min. Oocytes which had progressed from metaphase II arrest to anaphase II or telophase were scored as activated. Following culture, there was no observable difference in the number of eggs which resumed meiotic cell division in the control eggs (31 out of 47 oocytes activated, Fig. 5A) compared with PI-PLC treated eggs (27 out of 44 oocytes activated, Fig. 5B).

Treatment of zona-free oocytes with PI-PLC does not effect the ability of beads coated with anti-α6β1 antibodies to bind to oocytes

A bead binding experiment was performed to determine if PI-PLC treatment of zona-free mouse oocytes affected the antibody recognition of a well characterized egg surface integrin. Fluorescent beads were coated with α6β1 antibodies and incubated with either untreated or PI-PLC treated oocytes. No difference was observed in the number of α6β1 antibody-coated beads bound per oocyte between the control (Fig. 6A) and treatment group (Fig. 6B). Minimal bead binding was observed when beads were coated an with equivalent concentration of an irrelevant antibody and incubated with untreated eggs (Fig. 6C). These results indicate that treatment of oocytes with PI-PLC does not affect the ability of anti-α6β1 antibodies to bind its cognate antigen on the oolemmal. Therefore, using the egg surface integrin α6β1 as a model protein, the interactions of a non-GPI-anchored protein are not compromised by treatment with the PI-PLC preparation. This finding supports the hypothesis that the inhibitory effect of PI-PLC on fertilization was specifically due to the release of one or more GPI-anchored proteins from the oolemma following PI-PLC treatment and not to a non-specific perturbation of molecules on the surface by either PI-PLC or a contaminating substance in the enzyme preparation.

Treatment of Biotinylated Oocytes with PI-PLC Releases GPI-Anchored Proteins

Results from the in vitro assays led to the hypothesis that treatment of the oolemma with PI-PLC released one or more functionally relevant GPI-anchored protein(s) from the mammalian egg surface. Two-dimensional gel electrophoresis was then utilized to determine if the putative PI-PLC releasable proteins could be resolved and visualized. For these experiments 200 zona-free mouse oocytes were biotinylated, washed six times, separated into two groups, and incubated with or without 1U/ml PI-PLC for 30 min. Following six washes, egg proteins were extracted. The supernatants from the treated eggs as well as the egg extracts were separated by 2D electrophoresis, electroblotted to nitrocellulose membranes, probed with strepavidin-HRP, and biotinylated proteins were visualized on radiograms using enhanced chemi-luminescence. The 2D gel repertoire of biotin-labeled egg surface proteins present in the extracts of control eggs is presented in Fig. 7A. Approximately 20 biotin labeled proteins were resolved ranging in molecular weight from approximately 35 to 120 kDA with isoelectric points from 4.5 to 5.5. Following PI-PLC treatment of the oocytes (Fig. 7C), a decrease in the presence of the 70 kDa (pI 5) and 35-45 kDa (pI 5.5) protein clusters indicated by arrows in Fig. 7A was observed in extracts of eggs. The three spots denoted by asterisks in Fig. 7A represent proteins that bound strepavidin-HRP non-specifically and were detected on 2D blots of oocytes which were not biotinylated. 2D gel analysis of supernatant collected from untreated oocytes following 30

min of incubation showed that no biotinylated egg surface proteins were released into the medium (Fig. 7B). The repertoire of biotin-labeled proteins remaining on the egg surface following PI-PLC treatment is shown in Fig. 7C. Arrows denote the 70 kDa (pI 5) and 35-45 kDa (pI 5.5) protein clusters which were prominent in the extracts of untreated eggs (Fig. 7A) but decline in intensity in the extracts of eggs treated with PI-PLC. In contrast to the supernatant from untreated eggs (Fig. 7B), the supernatant from eggs treated with PI-PLC (arrows, Fig. 7D) revealed prominent 70 kDa and 35-45 kDa protein clusters of similar molecular weights and isoelectric points to those released from the eggs surface following PI-PLC treatment (Fig. 7C). It is likely that these GPI-anchored egg surface proteins are required for sperm-egg binding and fusion. Two minor protein spots at approximately 75-78 kDA and pI 5.5 (arrowheads, Fig. 7D) were also released from the egg surface into the supernatant following PI-PLC treatment. However, these proteins were only seen in two of five replications of this experiment.

Acid Tyrodes was used to remove the zona pellucida prior to cell surface biotinylation in the majority of the blotting experiments while chymotrypsin was used to de-zonulate oocytes for the sperm-egg binding and fusion assays. To ensure that the method of zona removal did not alter the pattern of labeled egg surface and PI-PLC released proteins, chymotrypisin was also used to de-zonulate oocytes prior to surface labeling and avidin blotting. Other than substituting chymotrypsin to de-zonulate oocytes, all conditions for this experiment were identical to the previous experiment using acid Tyrodes. The repertoire of surface labeled proteins present in the extracts of control eggs is shown in Fig. 8A. As with the previous experiment employing acid Tyrodes, there were 70 kDa (pI 5) and 35-45 kDa (pI 5.5) protein clusters present in the extracts of eggs which were not treated with PI-PLC (denoted by arrows in Fig. 8A). These proteins declined in intensity in the extracts of eggs treated with PI-PLC (denoted by arrows in Fig. 8C). Protein clusters of similar masses and isoelectric points to those which declined in intensity following PI-PLC treatment were observed in the supernatants from PI-PLC treated oocytes (denoted by arrows in Fig. 8D). No surface labeled proteins were seen in the supernatant from oocytes which were not treated with PI-PLC (Fig. 8B). Thus, PI-PLC releases 70 kDa and 35-45 kDa protein clusters regardless of the method of zona removal increasing confidence in the reproducibility of our findings.

6.4 DISCUSSION

The Effect of PI-PLC on Sperm-Egg Binding and Fusion is Mediated at the Level of the Oolemma

The results of the fertilization studies using zona-intact oocytes demonstrated that fertilization in vitro is blocked when mouse sperm and zona-intact oocytes are pre-

treated with PI-PLC prior to gamete co-culture in the presence of PI-PLC. The effect of the enzyme on fertilization does not appear to be at the level of sperm-zona pellucida interaction because PI-PLC had no significant effect on sperm-zona binding and significant numbers of perivitelline sperm were observed in the PI-PLC treated group following overnight incubation. These results suggest that GPI-linked sperm surface proteins may not be essential for penetration of the zona pellucida in mice. Another important finding from the zona-intact fertilization studies was that when zona-intact oocytes are treated with PI-PLC and washed prior to incubation with untreated sperm, fertilization is blocked. This result implies that the effect of PI-PLC is mediated at the oolemma. We then used the zona-free sperm-egg binding and fusion assay to further establish which gamete was being affected by PI-PLC treatment. Results showed that PI-PLC treated and washed sperm could bind to and fuse with untreated zona-free oocytes at levels similar to controls. Therefore, the effect of PI-PLC on fertilization does not appear to be mediated at the level of the sperm surface. Previous investigators have treated mouse sperm with similar PI-PLC concentrations and found that numerous iodinated GPI-anchored proteins could be visualized on 1D gel autoradiographs of supernatants from treated sperm (Thaler and Cardullo, 1995, Biochem. 34, 7788-7795), therefore, the lack of effect seen in this study does not appear to be due to and insufficient enzyme concentration. Perhaps the most important observation of this study was that fertilization was blocked when either zona-intact or zona-free eggs were treated with PI-PLC and washed prior to incubation with untreated sperm. These results imply that one or more GPI-anchored oolemmal proteins are required for sperm-egg binding and fusion.

The Inhibitory Effect of PI-PLC on Fertilization is Specific to GPI-Anchored Proteins

As noted in the introduction several investigators have previously studied the effects of various preparations of phospholipase C (PLC) on fertilization. Most PLC has a broad specificity for a variety of phospholipids with only minor differences in efficiency of hydrolysis (Low et al., 1986, Biochem. J. 237: 139-145). Several phospholipases have additional specificity for particular phospholipid structures. The *B. thuringiensis*-derived recombinant PI-PLC is specific for phosphatidylinositol and glycosylated phosphatidylinositol phospholipids (Low et al., 1988, J. Imm. Meth. 113: 101-111) and for this reason was selected for investigating the role of GPI-anchored proteins in sperm-egg interaction in this study.

Due to the broad specificity of PLC, results from previous studies investigating the inhibitory effects of PLC on sperm-egg interaction (Hirao and Yanagimachi, 1978, Gam. Res. 1: 3-12; Boldt et al.,1988, Biol. Reprod. 39: 19-27) do not

directly address the question of whether oolemmal GPI-anchored proteins are involved in fertilization. Also, other investigators found that while relatively impure PLC preparations blocked sperm-egg fusion, 'purer' PLC preparations (including PI-PLC) did not affect sperm-egg fusion (Clark and Koehler, 1988, Gam. Res. 19: 339-348). The authors concluded that contaminants in the 'impure' PLC preparations disrupted oocyte morphology (as determined by electron microscopy) which in turn impaired the ability of sperm to fuse with the egg. Regarding the effects of treating oocytes with PI-PLC, the authors did report a slight, but significant, inhibition of sperm-egg fusion. In the (Clark and Koehler, 1988, Gam. Res. 19: 339-348) experiments, short treatment times (3 min) were used for both PLC and PI-PLC because of the disruptive effect of impure PLC preparations on the oocytes. However, while it is known that PLC can cause non-specific membrane perturbations in intact cells (Mollby et al., 1973, Toxicology 11: 139-147), the same phenomenon has not been reported for PI-PLC. Therefore, it is possible that longer treatment of hamster oocytes with PI-PLC might have generated a greater inhibitory effect on sperm-egg fusion. In fact, we have recently found that when zona-free hamster oocytes are treated for 30 min with 1U/ml of PI-PLC, washed, and incubated with untreated human sperm, binding and fusion is almost completely blocked. However, when human sperm are treated with PI-PLC, washed, and incubated with untreated zona-free hamster oocytes, binding is significantly enhanced, while fusion is not affected. The GPI-anchored proteins which are released from the hamster oolemma following PI-PLC treatment have also been visualized on 2D avidin blots (see Example in Section 7).

In the present study, a number of controls were performed to validate that the effect of PI-PLC on mouse oocytes was specifically due to the release of GPI-anchored proteins following PI-PLC treatment. When one μg of the PI-PLC preparation was separated by 2D electrophoresis and silver stained, only one prominent protein spot (~30 kDa) could be visualized. This spot corresponded to the anticipated mass of PI-PLC. To ensure that the effect of the enzyme on fertilization was not due to an inorganic component of the enzyme preparation, heat inactivated PI-PLC was used as a control for all in vitro assays. Further, the effect of PI-PLC on fertilization was dose-dependent. The dose-response curve trial indicated that the maximal effect of PI-PLC on sperm-egg binding and fusion was 1-5 U per ml; which corresponds to that which is recommended for maximal release of GPI-anchored proteins (Dr. Martin Low, personal communication). Importantly, oocytes which had been treated with PI-PLC could be artificially activated with calcium ionophore, indicating that treated oocytes remained functionally viable. Finally, beads coated with $\alpha 6\beta 1$ antibodies could bind to PI-PLC treated eggs in a manner similar to that of controls, thus indicating that non-GPI-linked oolemmal proteins did not appear to be

affected by the PI-PLC preparation. Taken together, the in vitro data demonstrate that one or more oolemmal GPI-anchored protein(s) are required for fertilization and release of these proteins from the oolemma following PI-PLC treatment prevents oocytes from being fertilized by sperm.

Resolution of the Repertoire of Mammalian Oolemmal Surface Proteins Using Two-Dimensional Gel Electrophoresis and Cell Surface Vectorial Labeling

Although several investigators have surface labeled oocytes and visualized the proteins on 1D blots, there are no previous studies which utilize two-dimensional gel electrophoresis to visualize surface-labeled oolemmal proteins. Boldt et al. (1989, Gam. Res. 23: 91-101) radioiodinated zona-free mouse eggs and found that there were 8-10 egg surface proteins that incorporated iodine with major bands at 145-150, 94 and 23 kDa. (Flaherty and Swann, 1993, Mol. Reprod. Dev. 35: 285-292) evaluated the oolemmal protein pattern of biotinylated zona-free mouse eggs and found that there were two predominant bands of 82 and 69 kDa, eight major bands, and 14 minor bands which could be visualized. Recently, (Ya Zhong et al.,1997, Mol. Reprod. Dev. 47: 120-126) investigated the pattern of human, hamster and mouse oolemmal proteins after biotinylation and found that the overall staining pattern is similar between species. In human oocytes, these investigators identified 13 biotinlyated protein bands of which a 71kDa protein predominated. This study also observed the changes in the pattern of oolemmal protein expression during oocyte maturation. The total number of membrane proteins decrease from germinal vesicle to MII stage oocytes, while during this same time period, the relative proportion of the 71 kDa band increased from 9.9% to 27%.

In the present study, the repertoire of murine oolemmal surface proteins has, for the first time, been resolved using two-dimensional gel electrophoresis and cell surface biotinylation. Results from the 2D avidin blots (Fig. 7A) demonstrate that there appear to be a limited number (~ 20) of biotinylatable surface proteins on the mouse oolemma with a predominant protein at ~70 kDa (indicated by the top arrow in Fig. 7A). Further, this study has also indicated that the method of zona removal did not significantly alter the 2-D repertoire of oolemmal proteins. We found that, while there are some differences, the overall surface labeling patterns are similar when comparing acid Tyrodes de-zonulated oocytes (Fig. 7) with chymotrypsin de-zonulated oocytes (Fig. 8). The majority of surface-labeled proteins detected are acidic, with approximate isoelectric points ranging between 4.5 and 5.5. The resolution of this cohort of egg surface proteins by 2-D gel electrophoresis provides a basis for proceeding with the microsequencing, identification, and cloning of unknown proteins from oocytes.

Characterization of GPI-Anchored Proteins Using Two-Dimensional Gel Electrophoresis

The ability to visualize a repertoire of biotinylated oolemmal proteins on 2D avidin blots then allowed us to determine if treatment of biotin-labeled oocytes with PI-PLC releases oolemmal proteins from the egg surface into the supernatant. Comparison of PI-PLC treated and untreated eggs as well as the egg supernatant proteins from these groups reveal a predominant 70 kDa and less prominent 35-45 kDa protein cluster (indicated by arrows in Fig. 7A) to be released from the oolemmal surface into the supernatant following PI-PLC treatment (Fig.7C and Fig. 7D).

When analyzing the released protein(s) in (Fig. 7D) both the 70 kDa and the 35-45 kDa protein clusters appear as a series of closely aligned columns having slightly different isoelectric points. Previous literature on 2-D gel protein patterns (Shackelford et al., 1980, J. Exp. Med. 151: 144-165); (Negm et al., 1991, Comp. Biochem. Phys. Comp. Biochem. 99: 741-749) suggest that these protein clusters represent isoforms of a glycosylated protein. It is not known at this time whether the 70 kDa and 35-45 kDa protein clusters are in fact different proteins or are different isoforms of the same protein. While the 70 kDa and 35-45 kDa protein clusters represent candidate mediators of sperm-egg binding and fusion we have no direct evidence as yet they are, in fact, required for fertilization.

Possible Role of the GPI-Anchored Proteins in the Fertilization Cascade

The precise function of these PI-PLC sensitive proteins in the fertilization process is unknown at this time. One possibility is that the molecules are involved in the block to polyspermy as is the Ascidian GPI-anchored molecule, N-acetylglucosaminidase (Lambert, 1989, Development 105: 415-420). In Ascidians, sperm first bind to the ligand, N-acetylglucosamine, on the vitelline coat (VC), then penetrate through the VC and the perivitelline space to reach the egg surface, where sperm-egg fusion then occurs (Rosati and De Santis, 1980, Nature 283: 762-764). Ascidian eggs release N-acetylglucosaminidase from the cell surface into the sea water immediately following fertilization (Lambert, 1989, Development 105: 415-420). The enzyme can also be released by treating Ascidian eggs with exogenous PI-PLC (Lambert and Goode, 1992, Dev. Biol. 154: 95-100). Upon release, N-acetylglucosaminidase then modifies N-acetylgucosamine on the vitelline coat, thus preventing subsequent sperm penetrations. The investigators hypothesize that N-acetylglucosaminidase is released from the egg surface by the activity of endogenous phospholipases operating on the extracellular side of the surface (Goode et al., 1997, Dev. Growth. Diff. 39: 655-660).

In mice N-acetylglucosaminidase is also released from oocytes following fertilization (Miller et al., 1993, J. Cell. Biol. 123: 1431-1401) and subsequently occupies the sperm β1,4-Galactosyltransferase binding site on the zona pellucida, thus preventing polyspermy. While the molecular weight of mouse N-acetylglucosaminidase has yet to be determined, in Ascidians the released protein exhibits bands at 62 and 70 kDa (Lambert and Goode, 1992, Dev. Biol. 154: 95-100). It seems unlikely that the PI-PLC sensitive oolemmal protein in the present study is a GPI-anchored form of N-acetylglucosaminidase because sperm-zona binding was not affected by treating zona-intact eggs with PI-PLC. Further, N-acetylglucosaminidase does not appear to be GPI-anchored and has been localized to the cortical granules in mice (Miller et al., 1993, J. Cell. Biol. 123: 1431-1401) and therefore would not be susceptible to PI-PLC treatment.

Investigators have shown that a block to polyspermy also exists in mammals at the level of sperm-oolemma binding and fusion (Horvath et al.,1993, Mol. Reprod. Dev. 34: 65-72), however, the mechanism by which this block occurs is unknown. Results from this study demonstrate that when oocytes are treated with PI-PLC, fertilization is blocked and GPI-anchored protein(s) are released from the oolemma. One model to explain the observed results would posit that the GPI-anchored protein(s) performs a dual role as an oolemmal receptor for sperm and also mediates the oolemmal block to polyspermy. In this model when sperm bind to the GPI-anchored sperm receptor during the initial sperm-oolemmal interaction, second messenger pathways might then be activated which would result in the rapid release (possibly by endogenous PLC) of the remaining GPI-anchored sperm receptors from the oolemmal surface, thus preventing subsequent sperm from binding to the oolemma. If this hypothesis were correct, then treatment of oocytes with exogenous PI-PLC in this study could have blocked sperm-egg binding and fusion by releasing the GPI-anchored sperm receptors prior to sperm-egg interaction. Experiments are currently underway to isolate, characterize, and clone the released protein(s) and investigate their role in fertilization.

In conclusion, in vitro fertilization studies reported here demonstrate that the treatment of mouse sperm with PI-PLC does not affect the ability of sperm to penetrate the zona pellucida or to the ability of sperm to bind to and fuse with the oolemma. However, treatment of mouse oocytes with PI-PLC dramatically reduces the oocytes ability to bind to and fuse with sperm. The effect of PI-PLC on fertilization was: dose-dependent; PI-PLC did not alter the ability of treated eggs to be artificially activated; and did not effect the ability of beads coated with α6β1 integrin antibodies to bind the oolemma. 2D gel experiments demonstrate that 70 kDa (~ pI 5) and 35-45 kDa (~pI 5.5) protein clusters are

released from the oolemma following PI-PLC treatment. It seems likely that the released GPI-anchored protein(s) are required for fertilization.

7. EXAMPLE: Characterization of a PI-PLC-sensitive Oolemmal Protein Mediating Human Sperm-Hamster Egg Binding and Fusion

In the Example presented herein, the effects of phosphatidylinositol-specific phospholipase C (P1-PLC) on human sperm-hamster egg interaction were investigated to determine if glycosyiphosphatidylinositol (GPI) anchored proteins are involved in sperm-egg binding and fusion. Two-dimensional electrophoresis was used to visualize proteins released from hamster oocytes following P1-PLC treatment. For the binding and fusion assay, either sperm or eggs were treated with lU/mi PI-PLC for 30 min and washed prior to gamete co-incubation. Treatment of human sperm with PI-PLC significantly enhanced sperm-egg binding while having no effect on sperm-egg fusion. Treatment of zona-free hamster oocytes with P1-PLC blocked sperm-egg binding and fusion. In order to identify the oolemmal GPI-anchored proteins involved in fertilization, egg surface proteins were labeled with sulfo-NHS biotin and either mock treated or treated with PI-PLC. Egg protein extracts and egg supernatant proteins from each group were then analyzed by two-dimensional gel electrophoresis followed by avidin blotting. Comparison of blots demonstrated that a predominant biotinylated 25-40 kDa protein cluster (pI 5-6) apparent in the mock treated egg extract blot was absent in the PI-PLC treated egg extract blot. A protein cluster of identical molecular weight and isoelectric point as the predominant 25-40 kDa protein cluster was observed in the P1-PLC supernatant blot while no proteins could be seen in the control supernatant blot. These results demonstrate that treatment of hamster oocytes with PI-PLC inhibits sperm-egg interaction and releases a 25-40 kDa protein cluster (p1 5-6) from the oolemma. This released protein cluster represents an oolemmal GPI-linked surface protein(s) which is involved in human sperm-hamster egg interaction.

7.1 MATERIALS AND METHODS

PI-PLC Preparation

The phosphatidylinositol-specific phospholipase C preparation used for this study was purchased from Boehringer Mannheim (Indianapolis, IN). The PI-PLC was isolated from the cultured filtrate of *Bacillus cerus* and migrates as a single band on an SDS-PAGE gel at 29,000 Daltons.

Sperm Penetration Assay

Gamete Preparation - Gamete incubations were carried out in microdrops under paraffin oil at 37°C and 5% CO_2. Ejaculated human semen was allowed to liquefy for at least 30 min. Five hundred µl of the ejaculate was placed under 2 ml of BWW medium (Irvine Scientific, Santa Ana, CA) with 5 mg/ml human serum albumin (HSA, Sigma) for 30 min. and the sperm were allowed to swim up. The swim up sperm were then washed twice by centrifugation (8 min at 600 g) in 10 ml volumes of BWW in 15 ml centrifuge tubes. The sperm were capacitated overnight in 250 µl microdrops of BWW with 30 mg/ml HSA at a concentration of 20 x 10^6 sperm/ml. Cumulus-oocyte complexes were collected from superovulated Golden Syrian hamsters and placed in BWW with 5 mg/ml HSA. Cumulus cells were removed by treating eggs with 1 mg/ml hyaluronidase (Sigma) for 3 min. Oocytes were then washed (for this treatment and all subsequent treatments) by passing the eggs through 20 µl drops of media covered with mineral oil using a pulled, heat polished, Pasteur pipette. Zonae pellucida were removed by treating eggs with 1 mg/ml trypsin (Sigma) for 30 sec followed by 5 washes.

Treatment of human sperm with PI-PLC - Following overnight capacitation, 2 x 10^6 sperm were treated for 30 min. with either 1U/ml PI-PLC or 1U/ml of heat inactivated (95°C for 5 min) PI-PLC in 100 µl of BWW with 30 mg/ml HSA. The sperm were then washed twice by centrifugation in five ml volumes of BWW in 15 ml centrifuge tubes to remove the PI-PLC. Treated sperm were then added to untreated zona-free hamster oocytes (~12 per treatment group) at a concentration of 2 X 10^6 sperm/ml in 20 µl drops of BWW with 30 mg/ml HSA and the gametes were co-incubated for 3 h.

Treatment of zona-free hamster oocytes with PI-PLC - zona-free oocytes were treated for 30 min. with either 1U/ml PI-PLC or lU/ml of heat inactivated PI-PLC in 20 ml drops of BWW with 30 mg/ml HSA. The eggs were then washed through 5 microdrops and incubated with untreated human sperm at a concentration of 2 X 10^6 sperm/ml for 3 h.

Quantitation of sperm-egg binding and fusion - Following gamete co-incubation loosely bound sperm were removed from the oocytes by gentle pipetting. The eggs were then treated with 1mM acridine orange-3% in DMSO (Sigma) for 15 sec to stain the chromatin and washed through three 20 µl microdrops. To quantitate binding, the oocytes were placed between a microscope slide and an elevated cover slip, the oocytes were visualized at 200X using a light microscope (Zeiss Axioplan) and the number of sperm bound per oocyte was recorded. The number of sperm fused per egg was scored by counting the number of acridine orange-stained decondensed sperm heads within each oocyte using fluorescent microscopy.

Artificial activation of oocytes

In order to ensure that PI-PLC treated eggs remained viable following PI-PLC treatment, zona-free eggs were treated with either 1U/ml PI-PLC or 1U/ml heat inactivated PI-PLC for 30 min as described in the sperm penetration assay section. Following treatment, oocytes were preloaded with 1 μm Hoechst dye #33342 (Sigma) for 10 min to stain chromatin and washed three times. The oocytes were then activated by placing the eggs in 0.5 μM calcium ionophore A23187 (Sigma) for 5 min followed by three washes. The eggs were incubated for three hours and oocytes were observed as described in the sperm penetration assay section. The eggs were considered activated if they had advanced from metaphase II arrest to anaphase II or telophase II (with second polar body).

Two Dimensional Gel Electrophoresis

Hamster oocytes were collected and de-zonulated as described in the sperm penetration assay section. The zona-free eggs were then washed six times in BWW media containing 100 μg/ml polyvinylalcohol (PVA, Sigma), biotinylated with 2 mg/ml Sulfo-NUS biotin (Pierce, Rockford, IL) in BWW/PVA for 7 min at room temperature, and washed six times in BWW/PVA. The eggs were then split into two groups of 130 and either mock treated or treated with 1U/ml PI-PLC in 20 μl drops for 30 min. The supernatants were removed, the eggs were washed six times, and the oocytes and the oocyte supernatants were then frozen at -70°C in BWW/PVA containing protease inhibitors (Complete™, Boehringer Mannheim, Mannheim, Germany). The oocytes and supernatants were extracted in Celis lysis buffer containing 2% (v:v) NP-40, 9.8M urea, 100mM dithiothreitol (DTT), 2% ampholines (pH 3.5-10), and protease inhibitors for 30 min. at room temperature (Rasmussen et al., 1991, Electrophoresis 12: 873-882). Isoelectric focusing (IEF) was performed using the Mini-PROTEAN II tube cell (Bio RAD, Richmond, CA) apparatus and protocol with an ampholine mixture (Pharmacia Biotech, Uppsala, Sweden) of pH 3.5-5 (30%), 3.5-10 (40%), 5-7 (20%), and 7-9 (10%). The tube gels were placed on 12 % mini slab gels and the focused proteins were separated in the second dimension at 20 mA per gel. The proteins were then electroblotted to nitrocellulose membranes at 125 mA for 45 min. The membranes were then stained with Protogold for 10 min to visualize the egg proteins and washed briefly with water. Next, the membranes were blocked in PBS with 0.1% Tween and 5% dried milk for 30 min at room temperature, washed 1X in PBS/0.1% Tween, and probed with 20 μg/ml strepavidin-HRP (Pierce) for 30 min at room temperature. The blots were washed 3X in PBS/0.1% Tween (10 min per wash) and the biotinylated proteins were visualized using TMB as a substrate.

Statistical Analysis

The sperm penetration assay and egg activation assay were each repeated three times. Experimental and control group averages were reported as means +/- the standard deviation. Groups were compared using the students T test and differences were reported at the 0.05 level of significance.

7.2 RESULTS

Pre-treatment of sperm with PI-PLC significantly enhances human sperm-hamster egg binding while having no effect on sperm-egg fusion

When capacitated human sperm are treated with PI-PLC, washed and co-incubated with untreated zona-free hamster oocytes, there is a significant increase in the number of sperm bound to the oolemma (21.3 sperm per egg) when compared to the control group in which sperm were treated with heat inactivated PI-PLC (10.33 sperm per egg) (Fig. 8A). However, treatment of capacitated human sperm with PI-PLC, did not significantly effect sperm-egg fusion (1.7 sperm fused per egg) when compared to the control group (1.4 sperm per egg; Fig. 8B).

Pre-treatment of zona-free hamster oocytes with PI-PLC blocks human sperm-hamster egg binding and fusion

When zona-free hamster oocytes are treated with PI-PLC, washed and co-incubated with untreated capacitated human sperm, there is a significant decrease in the number of sperm bound to the oolemma (0.3 sperm per egg) when compared to the control group in which oocytes were treated with heat inactivated PI-PLC (21.9 sperm per egg; Fig. 9A). Similarly, PI-PLC treatment also significantly decreased sperm-egg fusion (0.1 sperm per egg) when compared to the control group (2.1 sperm per egg; Fig. 9B). This result indicates that the inhibitory effect of PI-PLC on fertilization is mediated at the oolemma and is in close agreement with results obtained using the mouse in vitro fertilization model (See Examples in Section 6).

Treatment of zona-free eggs with PI-PLC has no effect on artificial egg activation

Artificial activation of oocytes was performed to ensure that the oocytes remained viable following PI-PLC treatment. When eggs were treated with PI-PLC washed and artificially activated with 0.5 μm calcium ionophore A23187, there was no difference ($p < 0.05$) in the percentage of eggs which resumed meiotic cell division when comparing eggs treated with heat inactivated PI-PLC (84%, Fig. 10A) to eggs were treated with active PIPLC (83%, Fig. 10B). The observation that PI-PLC treatment did not alter meiotic

division supports the hypothesis that eggs remained viable following treatment and the PI-PLC effect on sperm-egg binding and fusion was authentic.

A 25-40 kDa (pI 5-6) Protein Cluster is Released from Ocytes treated with PI-PLC

Hamster oocytes were biotinylated and incubated with or without PI-PLC for 30 min. The supernatants were collected from the two groups, the eggs were washed, and the egg proteins were extracted. The egg protein extracts and the proteins from the supernatants were separated by 2-D electrophoresis and electroblotted to nitrocellulose membranes. The membranes were then stained with Protogold to visualize the egg proteins. Next the membranes were probed with strepavidin-HRP and the biotinylated egg surface proteins were visualized using TMB membrane peroxidase substrate. The repertoire of zona-free hamster egg proteins is shown in Fig. 11A with over one hundred egg proteins being resolved following Protogold staining (red staining). The repertoire of surface-labeled zona-free hamster egg proteins (blue staining) can also be seen in Fig. 11A. Approximately eleven biotinylated surface protein spots having molecular weights ranging from ~ 40 to 140 kDa can be visualized (small arrowheads). Seven of these surface labeled protein spots were also stained with Protogold (small arrowheads labeled d). One lesser ~ 45-50 kDa protein cluster c1), one predominant ~25-40 kDa protein cluster c2), and three protein trains having masses of ~ 35, 20 , and 15 kDa (t 1, 2, and 3 respectively) can be resolved. The predominant 25-40 kDa protein cluster c2) can be further resolved into three smaller protein clusters, however, the clusters were not recorded as separate proteins because continuous protein staining was observed between the clusters. The two spots denoted by asterisks in Fig. 10A represent proteins that bound strepavidin-HRP non-specifically and were detected on 2-D blots of oocytes which were not biotinylated.

The repertoire of biotin-labeled egg proteins remaining on the egg surface following PI-PLC treatment is shown in Fig. 11C. The two arrows denote the location of the 25-40 kDa (pI 5-6) protein cluster c2) which is prominent in the extracts of untreated eggs (Fig. 11A) but is absent the extracts of eggs treated with PI-PLC. Note that the staining intensity of the remaining surface labeled egg proteins does not appear to be affected by PI-PLC treatment. In contrast to the supernatant from untreated eggs (Fig. 11B), the supernatant from eggs treated with PI-PLC (arrows, Fig. 11D) reveal a 25-40 kDa (pI 5-6) protein cluster having a similar molecular weight and isoelectric point to that which was released from the egg surface following PI-PLC treatment (Fig. 11C). It is likely that the PI-PLC-sensitive protein cluster seen in Fig. 4D is GPI-anchored and is involved in human sperm-hamster egg binding and fusion. The train of four proteins having a mass of ~29 kDa and indicated by the asterisk in Fig. 11D most probably represents isoforms of PI-PLC

because identical staining patterns are observed when one μg of the PI-PLC preparation is separated on a 2-D gel and silver stained. It is possible that a small amount of unbound biotin remained associated with the biotinylated oocytes following oocyte washing and became linked to PI-PLC during oocyte treatment. This could explain why the PI-PLC isoforms appear to be dually labeled in Fig. 11D.

7.3 DISCUSSION

The findings presented herein demonstrate that PI-PLC has differing effects on human sperm and hamster eggs during gamete interaction. When human sperm were treated with PI-PLC, washed, and incubated untreated zona-free hamster oocytes, sperm-egg binding is significantly enhanced while fusion is not effected. GPI-anchored sperm surface proteins are thought to be involved in processes such as: protection of sperm from the immune response (Kirchhoff and Hale, 1996, Mol. Hum. Reprod. 2: 177-184); the acrosome reaction (Mendoza et al., 1993, J. Cell Biol., 121: 1291-1297); sperm-cumulus interaction (Myles and Primakoff, 1997, Biol. Reprod., 56: 320-327); and sperm-zona pellucida interaction (Mahoney et al., 1991, J. Reprod. Immunol. 19: 269-285; Diekman et al., 1997, Biol. Reprod. 57: 1136-1144). There are no reports in the literature implicating GPI-anchored sperm proteins in sperm-oolemma binding and fusion. In the studies of mouse egg proteins shown in the Example in Section 6, however, we found that when epididymal mouse sperm were treated with PI-PLC, washed, and incubated with untreated zona-free mouse oocytes, there was a slight (but not significant) increase in sperm-egg binding compared to controls, as shown in the Example in Section 6. It is possible that the enhanced increase in sperm-egg binding observed in the present study is due to the fact that ejaculated sperm were treated with PI-PLC as opposed to epididymal sperm which were used in the previous experiment.

One model to explain how treatment of sperm with PI-PLC could cause an increase in sperm-egg binding would posit that PI-PLC treatment releases GPI-anchored proteins from the sperm surface which mask molecules required for sperm-egg binding and fusion. It is known that sperm become coated with GPI-anchored proteins during passage through the epididymis (Kirchhoff and Hale, 1996, Mol. Hum. Reprod. 2: 177-184). It is possible that these proteins act as capacitating factors and are released from the sperm surface during passage through the female reproductive tract. Therefore, in this study, treatment of sperm with PI-PLC may have released more of these GPI-anchored capacitating proteins than were released from the control group, thus leading to enhanced sperm-egg binding. It is also possible that GPI-anchored sperm surface proteins are involved in acrosomal maintenance and loss of these proteins following PI-PLC treatment

increased the percentage of acrosome reacted sperm in the PI-PLC treatment group, thus leading to enhanced binding. Studies are currently underway to establish if PI-PLC affects capacitation or the acrosome reaction.

The most significant finding of this study is that when zona-free hamster oocytes are treated with PI-PLC, washed, and incubated with untreated human sperm, binding and fusion is blocked. While there are no previous reports describing GPI-anchored proteins on mammalian oocytes, there is a GPI-anchored form of N-acetylglucosaminidase which is present of the surface of Ascidian eggs (Lambert, 1989, Development 105: 415-420). This enzyme is PI-PLC-sensitive and is cleaved from the surface of Ascidians eggs following fertilization and occupies sperm binding sites on the vitelline coat to protect the egg against polyspermy (Lambert and Goode, 1992, Dev. Biol. 154: 95-100). Regarding the presence of GPI-anchored proteins on mammalian oocytes, two previous reports have investigated whether treatment of mammalian oocytes with PI-PLC blocks sperm-egg interaction. Clark and Koehier (1988, Gam. Res. 19: 339-348) treated zona-free hamster oocytes with up to 1 U/ml PI-PLC for only 3 min and found that the enzyme had a slight, but significant, inhibitory effect on hamster sperm-hamster egg fusion. However, these results are somewhat difficult to interpret due to the abbreviated treatment time. In our previous study we found that when either zona-intact or zona-free mouse oocytes were treated with PI-PLC, fertilization was blocked. To demonstrate that the effect of PI-PLC was specific to the release of GPI-anchored proteins, we also performed several control experiments. As with the hamster oocytes in this study, PI-PLC treated mouse oocytes are fully capable of being artificially activated, thus indicating that the oocytes are viable following treatment. Also, the decrease in mouse sperm-egg binding and fusion depended on the dose of PI-PLC employed, with a maximal inhibitory effect on binding and fusion at 1 U per ml. Finally, treatment of oocytes with PI-PLC did not reduce the immunoreactivity of the non-GPI-anchored egg surface integrin, $\alpha 6\beta 1$, as shown in the Example in Section 6. Therefore, in vitro data from our previous study as well as this study indicate that there is a PI-PLC-sensitive GPI-anchored protein(s) on the mammalian oocyte which is required for sperm-egg binding and fusion.

In this study, the repertoire of biotinylated hamster oolemmal proteins has been resolved using 2-D gel electrophoresis followed by avidin blotting. Results show that approximately eleven isolated protein spots, two protein clusters, and three protein trains are surface labeled (Fig. 11A). The two protein clusters (c1 and c2) represent separate proteins each with multiple isoforms containing varying degrees of glycosylation while the protein trains (t1, t2, and t3) represent non-glycosylated proteins each consisting of multiple isoforms (Shackelford et al., 1980, J. Exp. Med. 151: 144-165; Negm et al., 1991, Comp.

Biochem. Phys. Comp. Biochem. 99: 741-749). The number of surface labeled proteins which can be visualized on the 2-D avidin blots of hamster oocytes is consistent with results obtained from one dimensional blots of surface labeled mouse (Boldt et al., 1989, Gam. Res. 23: 91-101; Flaherty and Swan, 1993, Mol. Reprod. Dev. 35: 285-292; Ya Zhong et al., 1997, Mol. Reprod. Dev. 47: 120-126) and hamster (Ya Zhong et al., 1997, Mol. Reprod. Dev. 47: 120-126) oocytes. Also, although the 2-D repertoire of hamster oolemmal proteins is quite similar to that which was observed in the mouse, shown in the Example in Section 6, herein, there were notable differences in the masses of the protein clusters. In the mouse, the predominant oolemmal protein cluster is ~ 70 kDa with a less prominent protein cluster seen at 35-45 kDa. The hamster oolemma, on the other hand, contains a predominant protein cluster at ~25-40 kDa with a less prominent protein cluster at 45-50 kDa.

A most striking observation is that the predominant 25-40 kDa (pI 5-6) protein cluster (c2) which is evident in the 2-D avidin blots of untreated oocytes (Fig. 11A) is absent from the blot in which hamster oocytes were treated with PI-PLC (Fig. 11B). Further, the supernatant from eggs treated with PI-PLC (arrows, Fig. 11D) revealed a protein cluster of similar molecular weight and isoelectric point (25-40 kDa, pI 5-6) to that which was released from the eggs surface following PI-PLC treatment (Fig. 11D). The enzyme appears to be specifically affecting the 25-40 kDa protein cluster (c2) because the staining intensity of the remaining surface labeled proteins in Fig. 11C is similar to that which is seen in the untreated eggs in Fig. 11A. Of interest is the observation that, in the hamster, the predominant protein cluster (c2) is PI-PLC sensitive while the less prominent protein cluster (c1) is not affected by PI-PLC treatment (see Fig. 11B). However, in the mouse, both the 70 kDa and 35-40 kDa protein clusters are PI-PLC sensitive (see Example in Section 6).

In conclusion, when human sperm are treated with PI-PLC, sperm-egg binding is enhanced while sperm-egg fusion is not effected. When zona-free hamster oocytes are treated with PI-PLC, sperm-egg binding and fusion is blocked. Results from the 2-D avidin blots show that a predominant protein cluster is released from the hamster oolemma following PI-PLC treatment. This PI-PLC-sensitive protein cluster (~25-40 kDa, pI 5-6) is thus likely to mediate human sperm-hamster egg binding and fusion.

8. EXAMPLE: The Identification of a Novel Membrane-Bound Form of ZP-3

The experiments presented in the Example herein demonstrate the successful identification of a membrane-bound form of the zona pellucida sperm-binding protein, ZP3. ZP3 is a well known highly conserved, zona pellucida protein which has been characterized in many species and whose nucleotide and protein-coding sequences are known (for e.g., mouse GenBank Accession No. M20026; rat GenBank Accession No. Y10823; zebrafish AA566910; pig L22169; PCT patent no. WO9410304). The mRNA (SEQ ID NO:1) and protein (SEQ ID NO:2) sequence of human ZP3 is shown in Fig. 12. ZP3 is responsible for primary binding of the egg to a receptor(s) on the sperm (reviewed in McLeskey et al., 1998, Int. Rev. of Cytol. 177: 57-113). This binding event also initiates the acrosome reaction in which hydrolytic enzymes are released from the acrosomal compartment and act on the zona pellucida to facilitate penetration of the zona pellucida by sperm.

Because the GPI-linked M70 egg surface protein isolated from mouse eggs is similar to ZP-3 in size, the following experiment was carried out to determine whether M70 is a membrane-associated GPI-linked form of ZP3. Either zona-free mouse oocytes or mouse zona pellucidae were extracted in Triton-X114 and the detergent and aqueous phases were isolated. The samples were subjected to SDS-PAGE and Western blot analysis using the monoclonal antibody (mAb) (IE-10). As shown in Figure 13A, the oolemmal form of ZP3 partitions in the detergent phase. This indicates that oolemmal ZP3 is associated with the membrane and contains a membrane-associated domain. On the other hand, as shown in Fig.13B, zona matrix AP3 partitions in the aqueous phase, indicating that this form of ZP3 is soluble. These results further show that the mass of oolemmal ZP3 is less than that of matrix ZP3.

In Fig. 14, localization of SP3 to the oolemma of zona-free mouse oocytes was demonstrated by immunostaining with ZP3 mAb . The zonae pellucida were removed from ovulated mouse oocytes by treatment with chymotrypsin followed by mechanical agitation. As a control, the eggs were first treated with 10μg/ml Hoechst dye, which stains chromatin in blue. The eggs were then incubated with either 20μg/ml rat IgG or purified anti-ZP3 mAb (IE-10), washed 3 times, and incubated with fluorescent (CY-3)-conjugated goat anti-rat secondary antibodies. As shown in Fig. 14, oolemmal ZP3, which is stained in red, is localized to the oocytes microvillar region of oocytes. Similar results were seen when the zonae were removed with either acid Tyrodes or mechanical shearing.

Next, in order to characterize the membrane-bound form of ZP-3, kidney cells were transfected with full length recombinant mouse ZP3 (Genbank Accession No. M20026). Mouse ZP3 was cloned into a mammalian expression vector by cleaving pZP3.4 (Ringuette et al., Dev. Biol. 127: 287-295) with HindIII and EcoRI and ligating the ZP3-

encoding fragment into a pcDNA3.1 expression vector. Kidney cells were transiently transfected with the resultant construct using Transit (Panvera, Madison, Wisconsin). The cells were fixed with paraformaldehyde, incubated with the anti-ZP3 mAb, IE-10, and a fluorescently labeled secondary antibody. As shown in Fig.15, punctate cell surface staining pattern can be seen on cells which expressed the cDNA, while cells not expressing ZP3 do not react with the IE-10 mAb. At higher magnification, as shown in the center panel, distinct types of punctate staining can be visualized. The absence of staining on cells which do not express ZP3, indicates that ZP3 is specifically associated with the plasma membrane and is not adventitiously binding to the cell surface following secretion into the culture medium.

In a final demonstration of the membrane-associated ZP3, a peptide (J peptide) corresponding to the IE-10 mAb epitope on ZP3 was synthesized. The IE-10 mAb was incubated with either no peptide or saturating concentrations of the peptide prior to incubation with zona-free mouse oocytes. The eggs were then washed and incubated with fluorescently tagged secondary antibody. Indirect immunofluorescence images show that the peptide blocked antibody-antigen recognition, indicating that the IE-10 mAb was specifically recognizing ZP3 on the oolemmal surface.

In conclusion, the experiments described in this Example demonstrate the existence of a membrane-anchored form of ZP3. These results, taken together with the release of a 70 kDa protein upon treatment of mouse oocytes with PI-PLC, suggests the existence of a GPI-anchored form of ZP3. This GPI-linked, membrane bound, ZP3 is a potentially powerful antigen that could be used as a contraceptive vaccine. In particular, a GPI-linked human ZP-3 antigen, comprising a GPI-linked ZP3, or fragment thereof, would be useful as a human contraceptive.

9. EXAMPLE: Identification of a Subset of Egg Proteins Which Localize to the Egg Surface

The following Example demonstrates the identification and characterization of egg surface proteins. In particular, a novel sequence of a novel egg surface protein has been identified. These sequences can be used as antigenic molecules for the compositions and methods of the present invention.

Two-dimensional gel electrophoretic analysis of one thousand zona-free mouse oocytes was performed to determine the extent to which oolemmal proteins could be resolved on silver stained 2-D gels. In this experiment, zona-pellucidae were removed from the ovulated mouse oocytes using acid Tyrodes and the egg proteins were solubilized in a

non-ionic detergent/urea lysis buffer. The proteins were separated by isoelectric focusing followed by polyacrylamide gel electrophoresis. The gel was then silver stained to visualize egg proteins. Over five hundred oocyte proteins can be visualized on this gel. The approximate isoelectric points (pI) of the proteins and the approximate molecular masses (kDa) of the proteins have been determined. Most of the egg proteins fall within a pI range of 5-6.

Two-dimensional gel electrophoresis followed by avidin blotting was utilized to determine if vectorially labeled mouse oolemmal proteins could be resolved and visualized. Eight hundred zona-free mouse oocytes were surface labeled with Sulfo-NHS biotin and extracted with a non-ionic detergent. The extracted egg proteins were separated by 2-D electrophoresis, electroblotted to nitrocellulose membranes, probed with streptavidin-HRP, and biotinylated proteins were visualized on radiograms using enhanced chemi-luminescence. The 2-D gel repertoire of biotin-labeled egg surface proteins is presented in Fig. 1B. At least fifty isolated protein spots can be visualized with most falling in the 35-100 kDA (pI 5-6) region of the blot. Of particular interest are the three large protein clusters centered at ~70 kDa (pI 5), 40 kDa (pI 5.5) and 27 kDa (pI 5). It is likely that these clusters represent isoforms of heavily glycosylated proteins. To our knowledge, this is the first report of surface-labeled oocyte proteins being resolved on 2-D blots.

To ensure that plasma membrane proteins were biotinylated and cytoplasmic proteins were not, the blot described above was stripped of streptavidin-HRP and re-probed with antibodies to tubulin and actin. No surface-labeling of these cytoskeletal proteins occurred, validating the surface-specificity of the labeling method. Tubulin antibody localized precisely to a spot which, while surrounded by avidin-staining, is devoid of any labeling. To ensure that strepavidin was specifically recognizing Sulfo-NHS biotinylated egg proteins, 2-D blots of non-biotinylated oocytes were probed with strepavidin-HRP. Two proteins in the unlabeled eggs bound streptavidin.

9.1 Microsequencing Surface-labeled Proteins

With the preliminary oocyte proteomic database in place, and a subset of egg surface labeled proteins identified, microsequence information was obtained from several of the surface-labeled proteins. For the microsequencing experiments, one hundred zona-free mouse oocytes were biotinylated, separated on 2-D minigels, and the surface-labeled proteins were visualized by avidin blotting. Concurrent with the surface-labeling experiment, similar numbers of oocytes were separated on 2-D minigels and silver-stained. Coordinates of several surface-labeled proteins directly matched those of silver-stained proteins indicating that these oocyte proteins localized to the cell surface. It is known that

some glycoproteins cannot be visualized by standard silver staining techniques. The heterogeneous nature of the 70 and 35-45 kDa protein clusters (double arrowhead pointing to the right) suggest that these proteins may be heavily glycosylated which may account for their lack of silver staining. Four putatively surface labeled proteins were chosen for microsequencing based on their consistent labeling with biotin after multiple surface-labeling experiments.

Microsequence data was obtained using LC-MS tandem mass spectrometry (Wilm *et al.*. 1996). To ensure that sufficient protein concentrations would be available for this sequencing experiment, proteins from approximately 1300 zona-free oocytes were separated on a 2-D minigel and the gel was Coomassie-stained. The selected surface-labeled proteins spots were then cored from the minigel and submitted to the Mass Spectrometry Laboratory for microsequence analysis.

The resulting microsequence information is summarized below:

TABLE 1

Protein Identification	Protein Coordinates	% of Known Sequence	Type of Membrane Attachment	First Time Identified in Mammalian Egg
1 = Novel	MW 120, pI 4.3	N/A	N/A	N/A
2 = Calnexin	MW 107, pI 4.4	6%	integral	yes
3 = HSP 78 (BIP)	MW 78, pI 4.82	30%	none reported	yes
4 = Calreticulin	MW 64, pI 4.2	37%	associated with KDEL docking protein	yes

Protein # 1 - Three peptide sequences were deduced from the CAD spectra of ions from the tryptic digest of Protein #1. Database searches using Sequest could not identify these peptides in any database sequence. To validate the authenticity of these peptide sequences, protein # 1 was cored from another Coomassie stained gel containing similar numbers of extracted oocytes and re-submitted to the Mass Spectrometry Lab for microsequencing and identical results were obtained. Therefore, protein # 1 is novel and a prime candidate for cloning and characterization.

TABLE 2

Peptide Sequences From Protein #1 (NOVEL)

Peptide No.	Measured Molecular Weight ($M+H^+$, Da)	Peptide Sequence by CAD[1] (calculated MW, $M+H^+$, Da)	Peptide Sequence from Database (calculated MW, ($M+H^+$, Da)
1	843.6 +2		SFSDFLK (843.4)
2	1023.8 +2	uninterpretable	
3	1364.0 +2	__XPEATG ---K $\overline{b2}$ = 169	
4	1369.6 +2	__SXVNVS---	

[1] X designates I or L which cannot be distinguished by low energy CAD,
M(o) designates oxidized M,
C* designates carbamidomethyl modified C,
_ indicates an unknown residue,
- - - indicates an unknown number of unknown residues.

Proteins # 2, 3, and 4 - Sequence obtained from protein spots 2. 3, and 4 corresponded to calnexin, HSP78 (BIP), and calreticulin respectively. These proteins fall into a class of proteins which bind calcium and function as molecular chaperones. Molecular chaperones modulate the folding and assembly of newly synthesized proteins and protein complexes (Wynn *et al.*, 1994, J. Lab Clin. Med., 124:31-36). Calnexin and its soluble homologue, calreticulin, specifically modulate glycoproteins (Oliver *et al.*, 1996, J. Biol. Chem. 271:13691-96). Many cell surface proteins are glycoproteins and exist as complexes of multiple subunits. These subunits are thought to be assembled by molecular chaperones in the endoplasmic reticulum (Van Leeuwen and Kearse, 1996, J. Biol. Chem. 271:25345-49). There is a growing amount of evidence that, in some cases, the chaperone proteins are not released from the plasma membrane complexes with which they are assembling and are carried to the cell surface where they may be engaged in alternate functions than those which they perform in the ER. As an example, BIP, calnexin, and calreticulin assist in assembly of the T-cell antigen receptor complex (TCR) in the ER (Van Leeuwen and Kearse, 1996, *supra*). In thymocytes, calnexin maintains its association with CD3 (a component of the TCR) and the complex is expressed on the cell membrane of immature thymocytes where it is thought to play a role in cell signaling (Wiest *et al.*, 1995, EMBO J. 14:3424-33).

The highly conserved heat shock protein (HSP) family has generally been thought to function intracellularly. However, recent evidence suggest that, in some instances, these proteins are also expressed on the plasma membrane. For example, in some

types of T-cells, HSPs, are thought to actually function as antigen presenting molecules (Multhoff *et al.*, 1995, Int. J. Cancer, 10:272-9). They are also present on the surface of T-cells and mononuclear cells which are undergoing apoptosis (Poccia *et al.*, 1996, Immunology, 88:6-12). HSPs have also been implicated in fertilization (Boulanger *et al.*, 1995a, J. Cell Physiol. 165:7-17). The gene encoding the egg plasma membrane receptor for sperm was cloned in the sea urchin and the extracellular domain of the receptor is similar to the HSP70 family of proteins (Foltz *et al.*, 1993, Zygote, 1:276-9). Heat shock proteins have also been found on the surface of sperm by our lab and others (Boulanger *et al.*, 1995b, *supra*). Regarding BIP specifically, one function of this molecule is known to bind to and assist in the finding that one of the surface-localized oocyte proteins was calreticulin was of particular interest. Following the acrosome reaction, calreticulin is expressed on the plasma membrane overlying the equatorial segment of sperm. Because sperm-egg fusion is thought to initiate in this region of sperm, it is possible that sperm surface calreticulin is involved in this aspect of gamete interaction.

Recently, the cell surface protein, Clq-R, was found to have almost complete amino acid sequence identity with calreticulin (Stuart *et al.*, 1997). Clq-R is a receptor for Clq, a component of the classical complement pathway and has been found on many cell types (Peterson *et al.*, 1997) including oocytes(Fusi *et al.*, 1991) and sperm (Bronson *et al.*, 1998). One known function of cell-surface calreticulin is to mediate adhesion to extracellular matrix proteins such as laminin and fibrinogen (Gray *et al.*, 1995; McDonnell *et al.*, 1996; White *et al.*, 1995). This adhesion process is thought to occur in association with integrins such as $\alpha 6\beta 1$ (Zhu *et al.*, 1997). Given calreticulin's implicated role in fertilization, surface localization on sperm following the acrosome reaction, association with integrins, and confirmed cell surface expression in other cell types, we decided to use calreticulin as a model protein and proceed to the next aim of this grant proposal for known proteins, which is to investigate the role of identified oolemmal proteins in fertilization using in vitro fertilization assays.

The specificity of the rabbit polyclonal antibody to full length recombinant calreticulin used for the subsequent assays was confirmed by probing a 2-D blot of zona-free mouse oocytes. The blot was first stained with Protogold to visualize oocyte proteins (Fig. 3A). Immunoblotting revealed that the calreticulin antibodies are highly specific and recognize only two oocyte proteins, calreticulin and calnexin (Fig. 3B, arrows). It is not surprising that the calreticulin polyclonal antibody also recognized calnexin because calnexin is the membrane bound homologue of calreticulin. However, because both proteins were recognized by the calreticulin antibody, it is understood that the results presented below could be due to antibody reactivity with either calreticulin, calnexin, or

both. Fig. 3C highlights the observation that both calreticulin and calnexin were surface-labeled with biotin. The oocyte cell surface localization of calreticulin was then confirmed by indirect immunofluorescence. A greater fluorescence staining intensity was observed on live zona-free oocytes that were incubated with fluorescent beads coated with a 1-50 dilution of calreticulin antisera when compared to oocytes incubated with beads coated with a similar dilution of normal rabbit sera.

The effect of calreticulin antibodies on sperm-egg interaction was then investigated in an in vitro fertilization assay. Cumulus-free zona-intact mouse oocytes were either not treated with antibodies or treated with a 1:50 dilution of normal rabbit sera or anti-calreticulin antibodies. The eggs were then washed and inseminated with untreated mouse epididymal sperm. Following overnight incubation, oocytes which had not undergone cleavage were scored as unfertilized and oocytes which bad cleaved were scored as fertilized. Significantly fewer ($p < 0.05$) oocytes underwent cleavage following incubation with calreticulin antibodies (1.3%) when compared to oocytes which were either not treated (68%) or treated with normal rabbit sera (75%).

It appears that the effect of calreticulin antibodies on fertilization occurs following sperm-egg fusion and prior to syngamy. Interestingly, calreticulin has been shown to modulate calcium transients following engagement of surface integrins (Coppolino *et al.*, 1997) and when calcium transients are blocked, fertilization becomes arrested following sperm-egg fusion in both sea urchins (Lee and Shen, 1998) and mice (Xu et al., 1994).

The invention is not to be limited in scope by the specific embodiments described which are intended as single illustrations of individual aspects of the invention, and functionally equivalent methods and components are within the scope of the invention. Indeed various modifications of the invention, in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

All references cited herein are incorporated by reference herein in their entireties for all purposes.

WHAT IS CLAIMED IS:

1. An antigenic molecule comprising an isolated egg surface protein covalently linked to glycosylated phosphatidylinositol moiety and having sperm binding activity.

2. An antigenic molecule of Claim 1, wherein the isolated egg surface protein has the following characteristics: 1) a molecular weight of 70kDa; 2) a pI of 5; and 3) the ability to be specifically released from the egg surface upon treatment with phosphatidylinositol-specific phospholipase C.

3. An antigenic molecule of Claim 1, wherein the isolated egg surface protein has the following characteristics: 1) a molecular weight of 1) a molecular weight between 35 and 45 kDa ; 2) a pI of 5.5; and 3) the ability to be specifically released from the egg surface upon treatment with phosphatidylinositol-specific phospholipase C.

4. An antigenic molecule of Claim 1, wherein the isolated egg surface protein has the following characteristics: 1) a molecular weight between 25 and 40 kDa; 2) a pI between 5 and 6; and 3) the ability to be specifically released from the egg surface upon treatment with phosphatidylinositol-specific phospholipase C.

5. An antigenic molecule of Claim 1 wherein the isolated egg surface protein comprises ZP3, or a fragment or analog thereof covalently linked to a glycosyl-phosphotidyinositol moiety.

6. An antigenic molecule of Claim 1 wherein the isolated egg surface protein comprises the amino acid sequence of SEQ ID NO:2, or a fragment or analog thereof covalently linked to a GPI moiety.

7. A method for preparing antiserum comprising an antibody to an egg surface protein, said method comprising:

(a) immunizing an animal with an egg surface protein or an immunogenic fragment thereof;

(b) obtaining serum from the immunized animal;

(c) screening the serum for the ability to bind to an egg surface protein or an antigenic fragment thereof; and

(e) recovering serum with said ability.

8. A purified antiserum produced by the method of Claim 7 which comprises a polyclonal antibody.

9. An antiserum specific to the antigenic molecule of Claim 1.

10. A method for preparing a monoclonal antibody to an egg surface protein comprising:

(a) immunizing an animal with an egg surface protein;

(b) obtaining antibody-secreting cells from the immunized animal;

(c) immortalizing the antibody-secreting cells obtained in step (b) to produce immortalized cells producing monoclonal antibodies;

(d) screening the immortalized cells for the ability of their secreted antibodies to bind to the egg surface protein, or to inhibit *in vitro* fusion and fertilization, or to bind to an egg surface protein or an antigenic fragment thereof; and

(e) recovering the antibody secreted by the immortalized cells with said ability.

11. A purified antibody produced by the method of Claim 6.

12. An isolated antibody specific to the antigenic molecule of Claim 1.

13. A method for screening a molecule for activity to modulate the level of egg surface protein activity comprising contacting cells with the molecule, and comparing the level of egg surface protein, mRNA or activity in cells contacted with the molecule to the amount of egg surface protein, mRNA, or activity, in cells not so contacted, wherein an increase or decrease in the amount of egg surface protein, mRNA, or activity in the contacted cells relative to the amount of egg surface protein, mRNA, or activity in the cells not so contacted indicates that the molecule has activity to modulate egg surface levels or activity.

14. A method for screening a molecule for the ability to interact with an egg surface protein comprising contacting the egg surface protein with one or more molecules under

conditions conducive to complex formation between the egg surface protein and the molecule, and recovering a molecule that binds specifically to the egg surface protein.

15. A method for modulating fertility comprising administering to a subject a therapeutically effective amount of any of the antigenic molecules of Claims 1-6.

16. A method for modulating fertility comprising administering to a subject a therapeutically effective amount of an antiserum of Claims 8-9.

17. A method for modulating fertility comprising administering to a subject a therapeutically effective amount of an antibody of Claim 11-12.

18. A method for modulating fertility comoprising administering to a subject a therapeutically effective amount of a molecule that modulates the activity of an egg surface protein.

FIG.1A

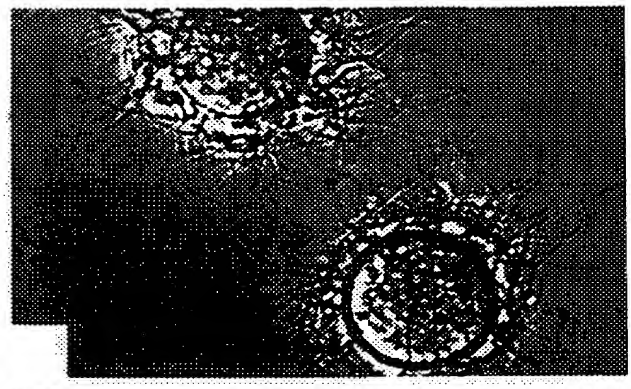
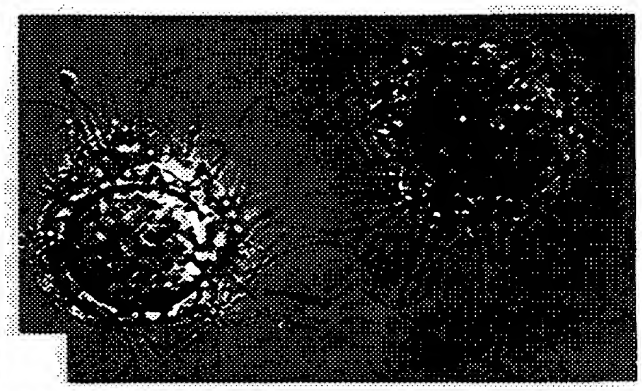


FIG.1B



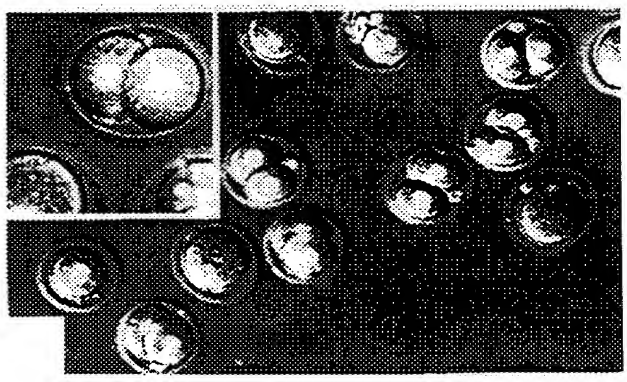


FIG.1C

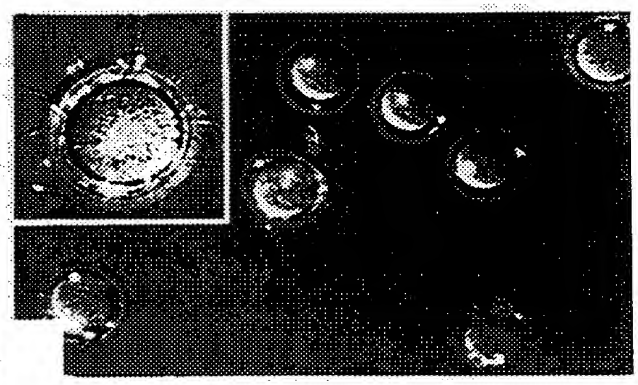


FIG.1D

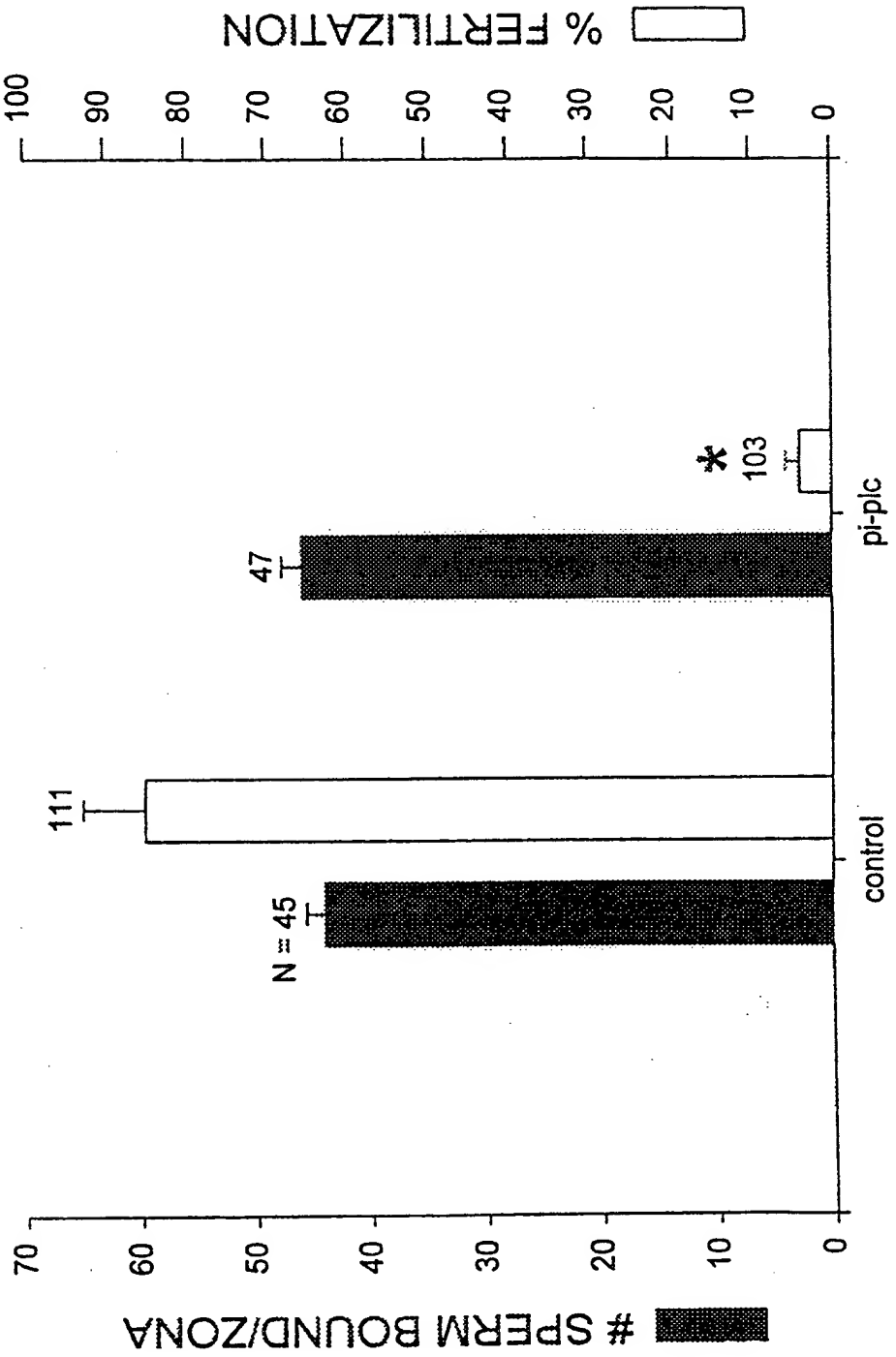

% FERTILIZATION
100
90
80
70
60
50
40
30
20
10
0
*
103
47
111
N = 45
pi-plc
control
SPERM BOUND/ZONA
70
60
50
40
30
20
10
0

FIG. 1E

FIG.2A

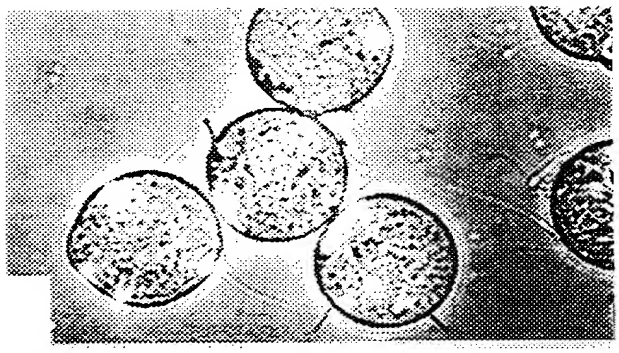
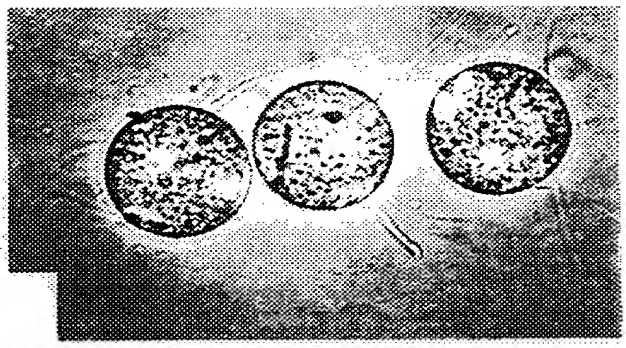


FIG.2B



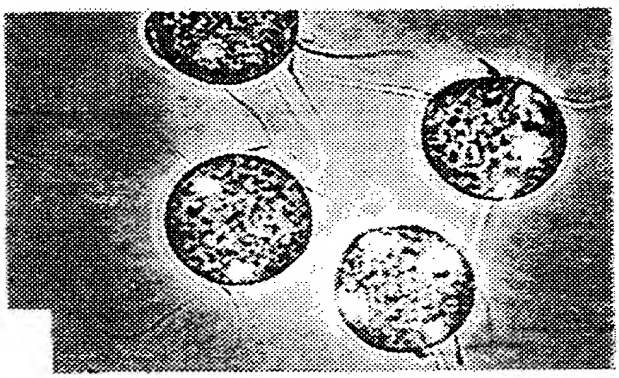


FIG.2C

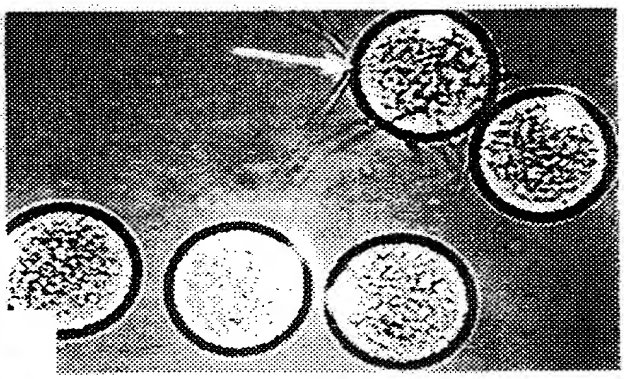


FIG.2D

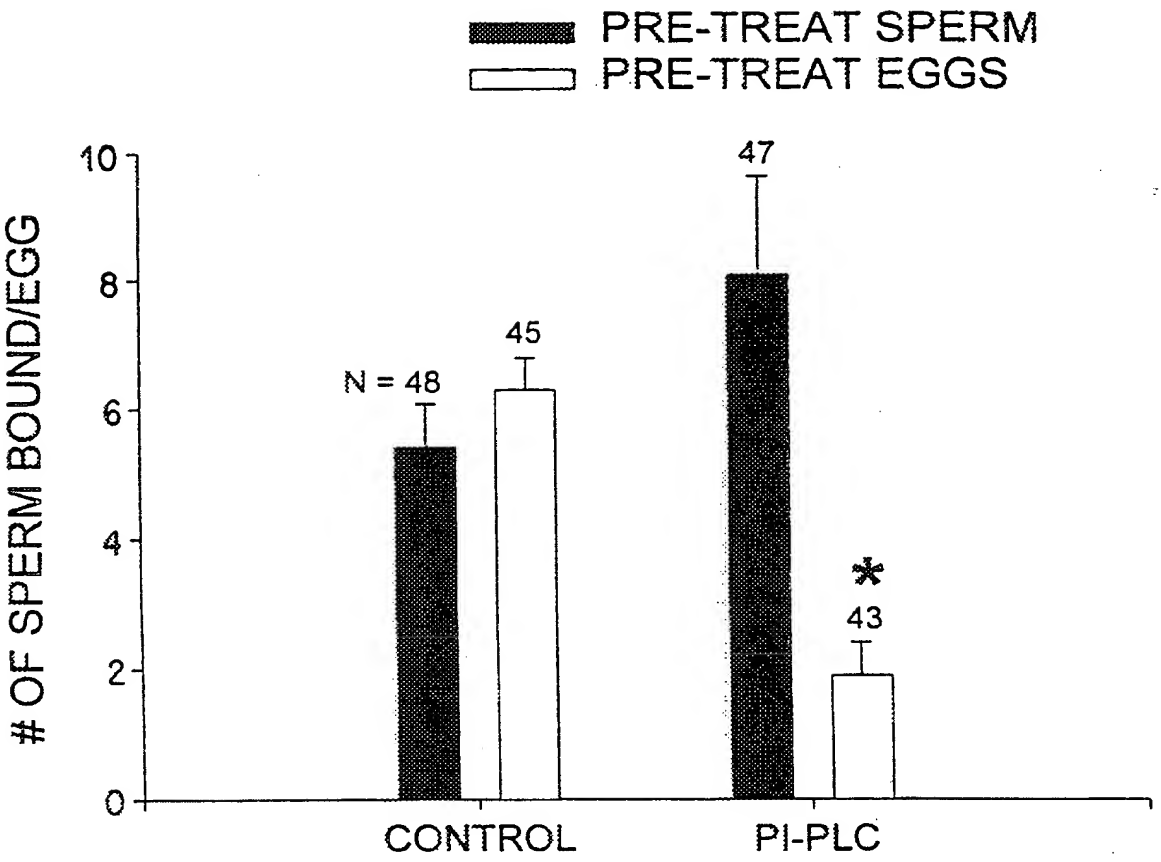

PRE-TREAT SPERM
PRE-TREAT EGGS
OF SPERM BOUND/EGG
10
8
6
4
2
0
N = 48
45
47
*
43
CONTROL
PI-PLC

FIG. 2E

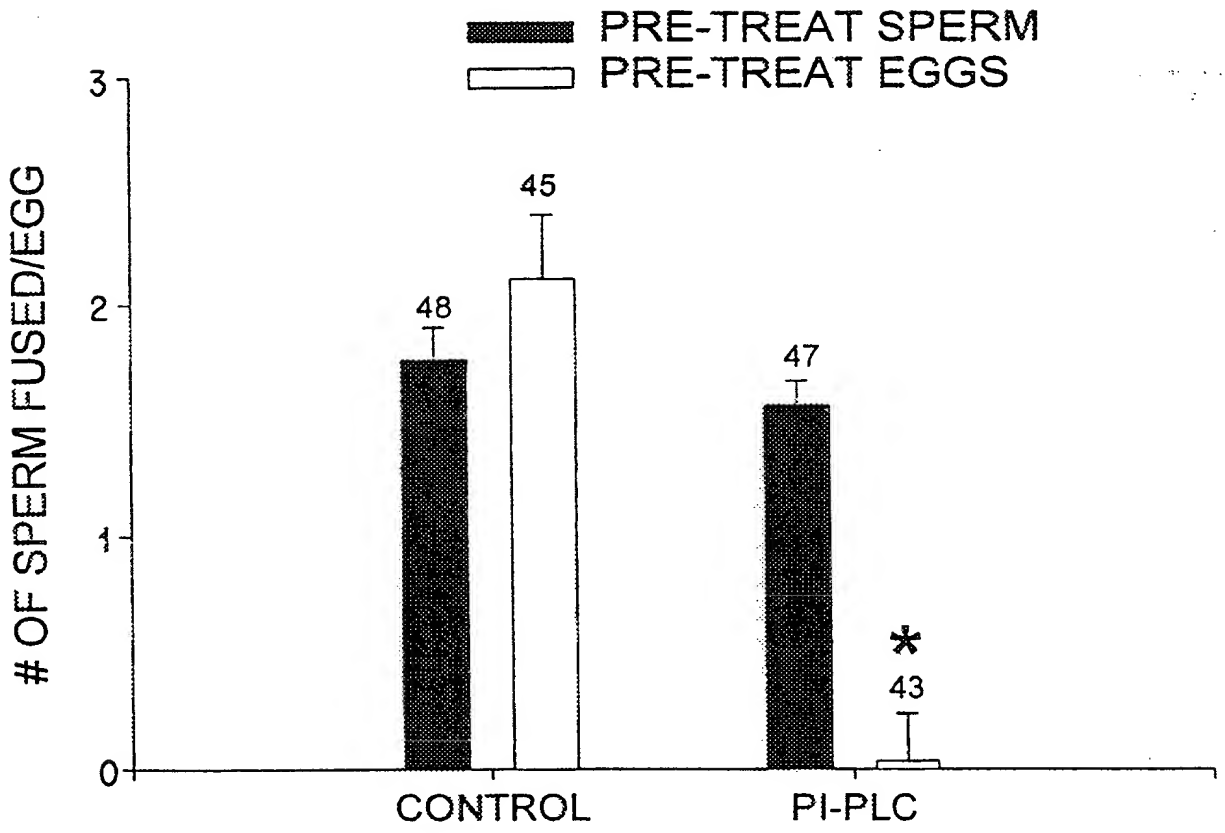

PRE-TREAT SPERM
PRE-TREAT EGGS
OF SPERM FUSED/EGG
3
2
1
0
48
45
47
*
43
CONTROL
PI-PLC

FIG. 2F

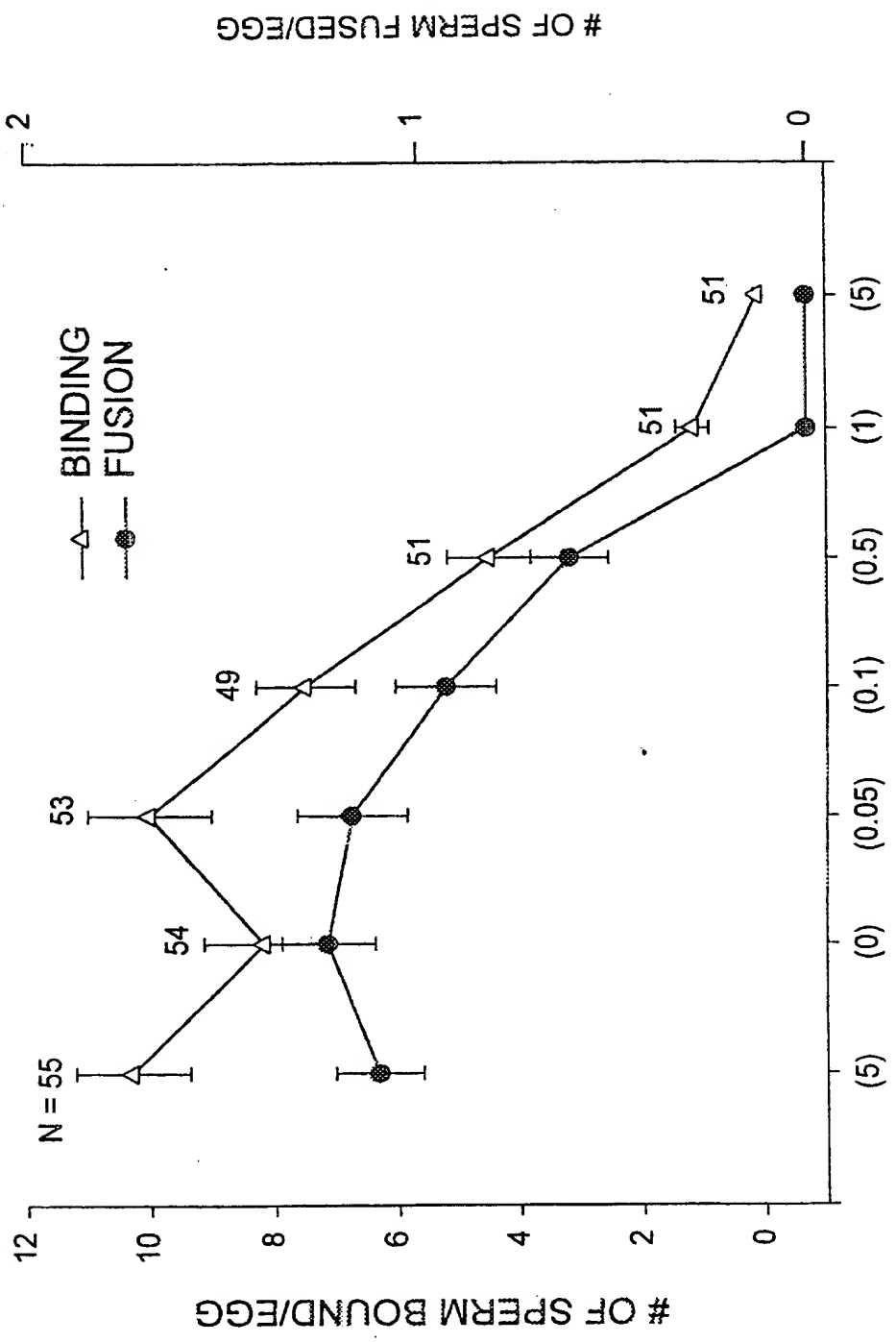

OF SPERM FUSED/EGG
2
1
0
BINDING
FUSION
N = 55
53
54
49
51
51
51
OF SPERM BOUND/EGG
12
10
8
6
4
2
0
(5)
HEAT INACTIVATED
(0)
(0.05)
(0.1)
(0.5)
(1)
(5)
PI-PLC CONCENTRATION (U/ml)

FIG. 3

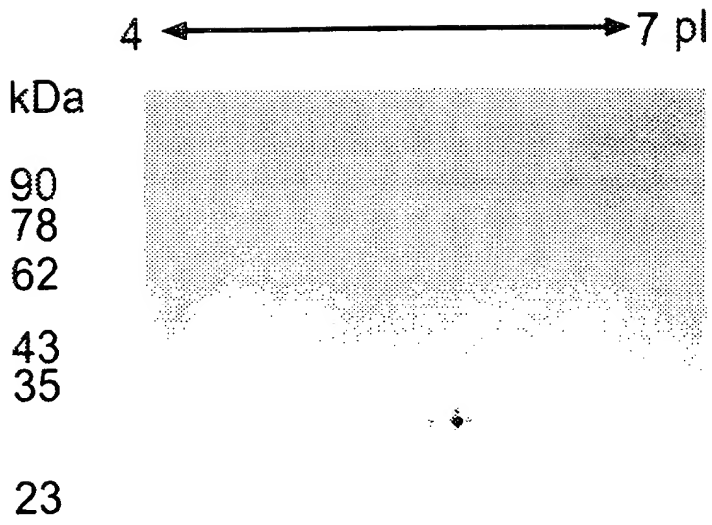


FIG.4

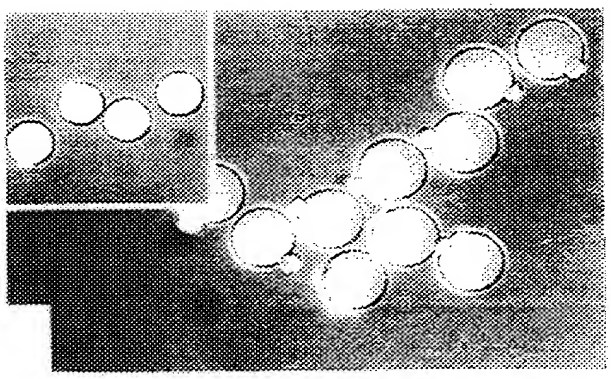


FIG.5A

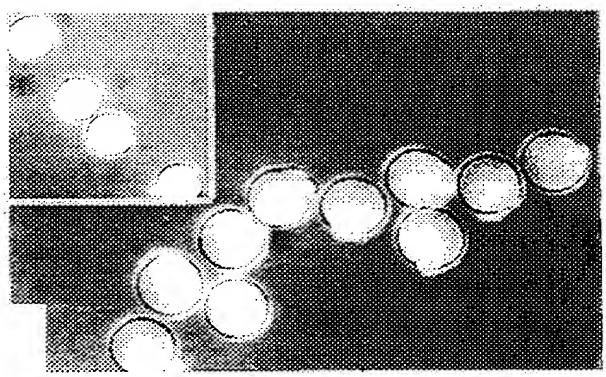


FIG.5B

FIG.6A

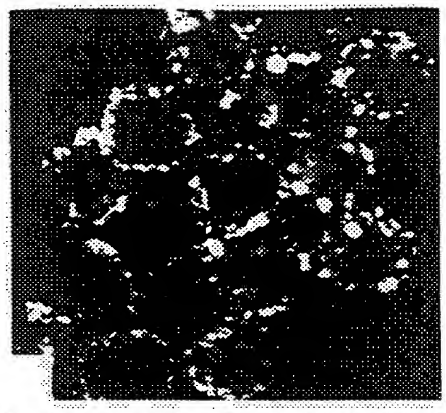


FIG.6B

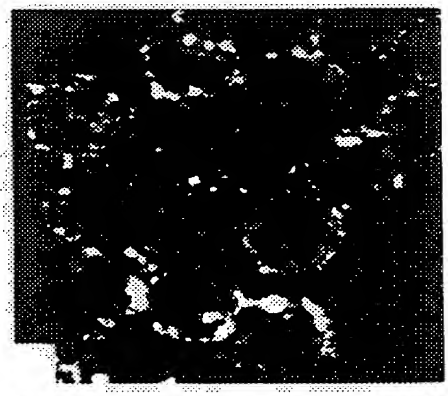


FIG.6C

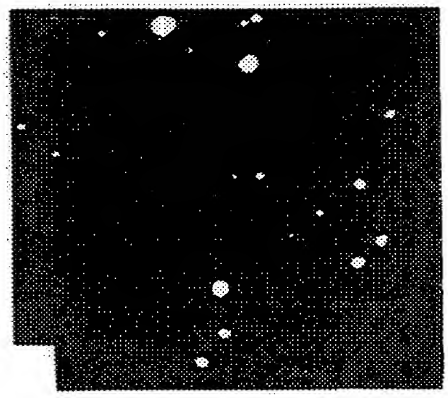


FIG.7A

FIG.7B

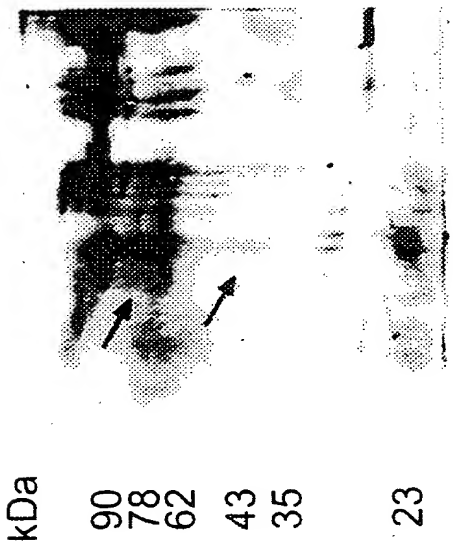


FIG.7C

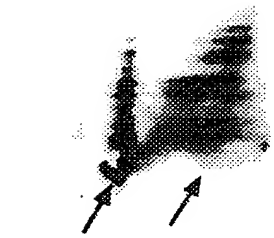


FIG.7D

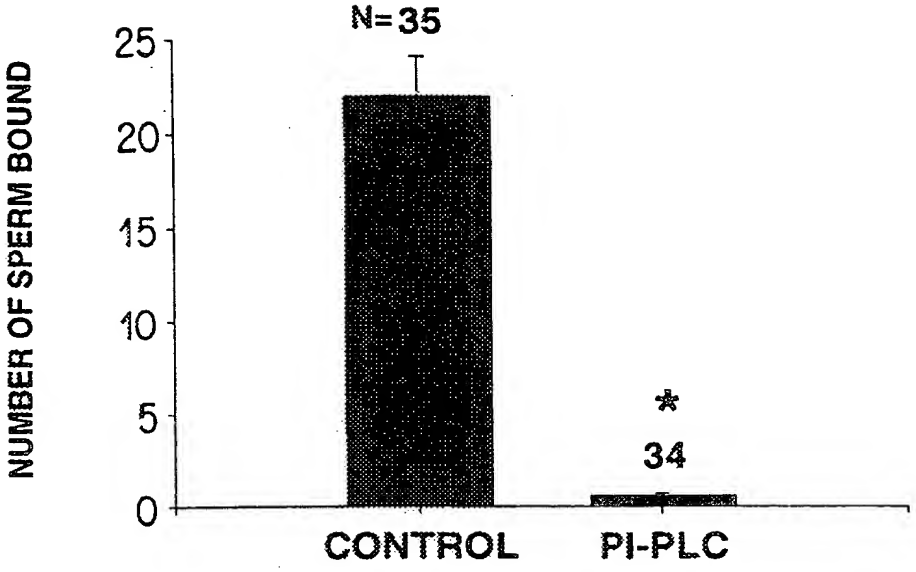

N=35
25
20
15
10
5
0
NUMBER OF SPERM BOUND
*
34
CONTROL
PI-PLC

FIG. 8A

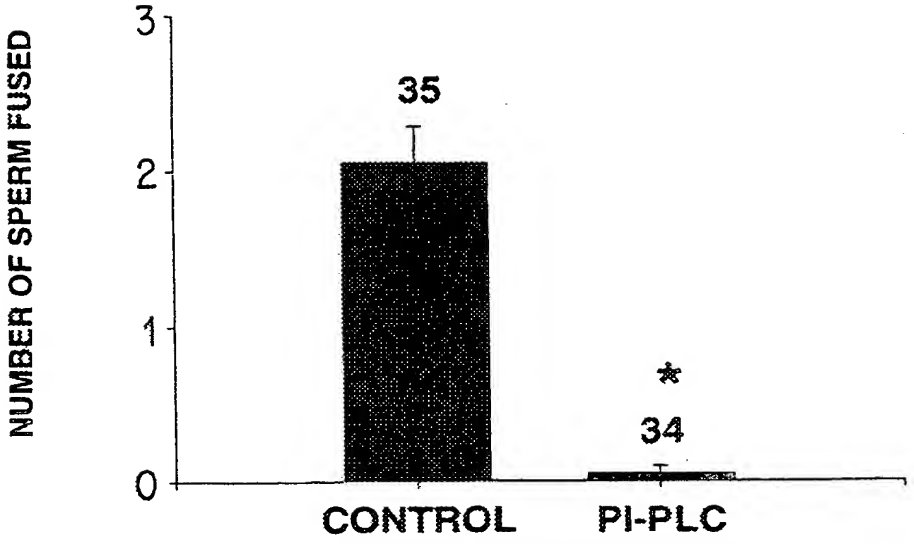

3
2
1
0
NUMBER OF SPERM FUSED
35
*
34
CONTROL
PI-PLC

FIG. 8B

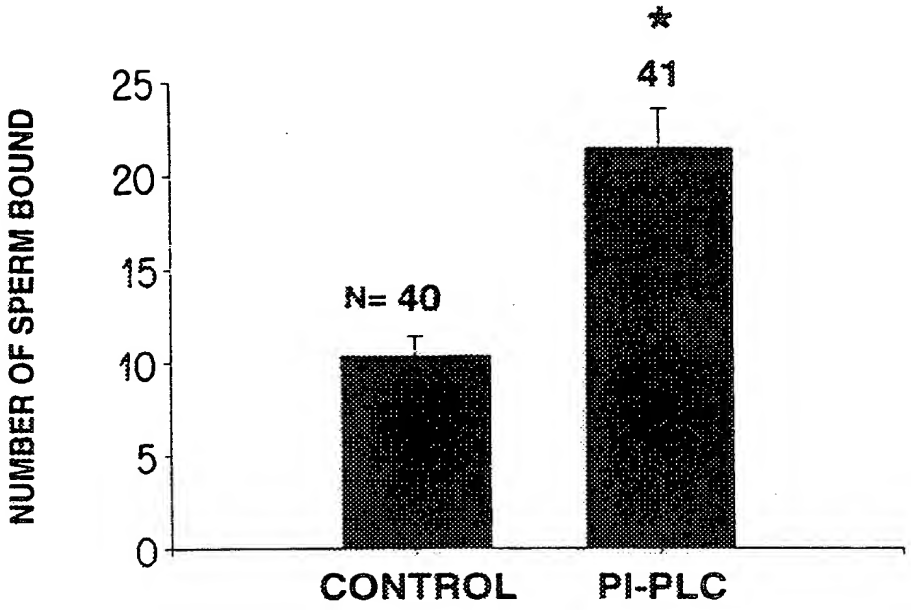

NUMBER OF SPERM BOUND
25
20
15
10
5
0
*
41
N= 40
CONTROL
PI-PLC

FIG. 9A

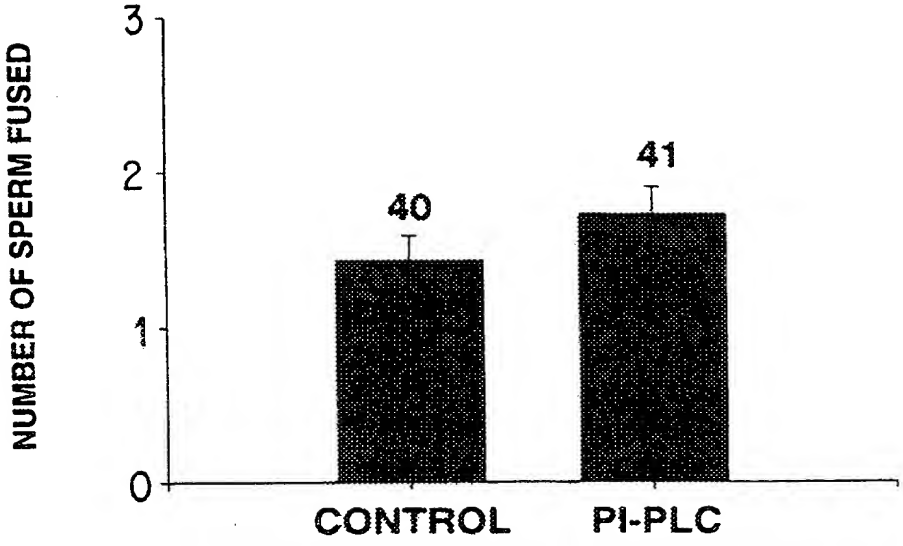

NUMBER OF SPERM FUSED
3
2
1
0
41
40
CONTROL
PI-PLC

FIG. 9B

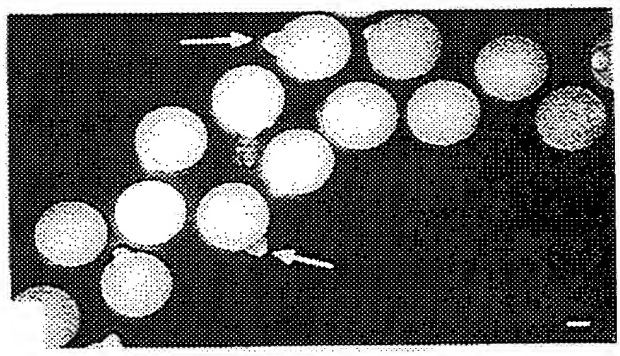


FIG.10A

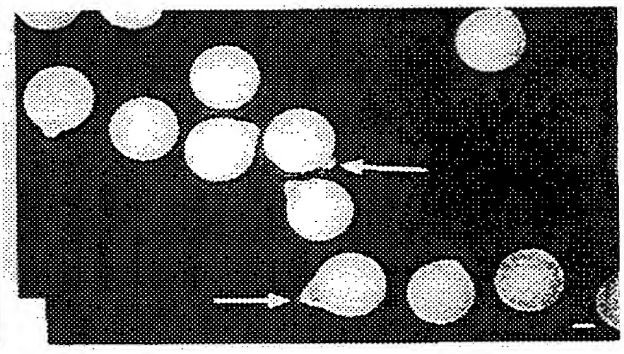


FIG.10B

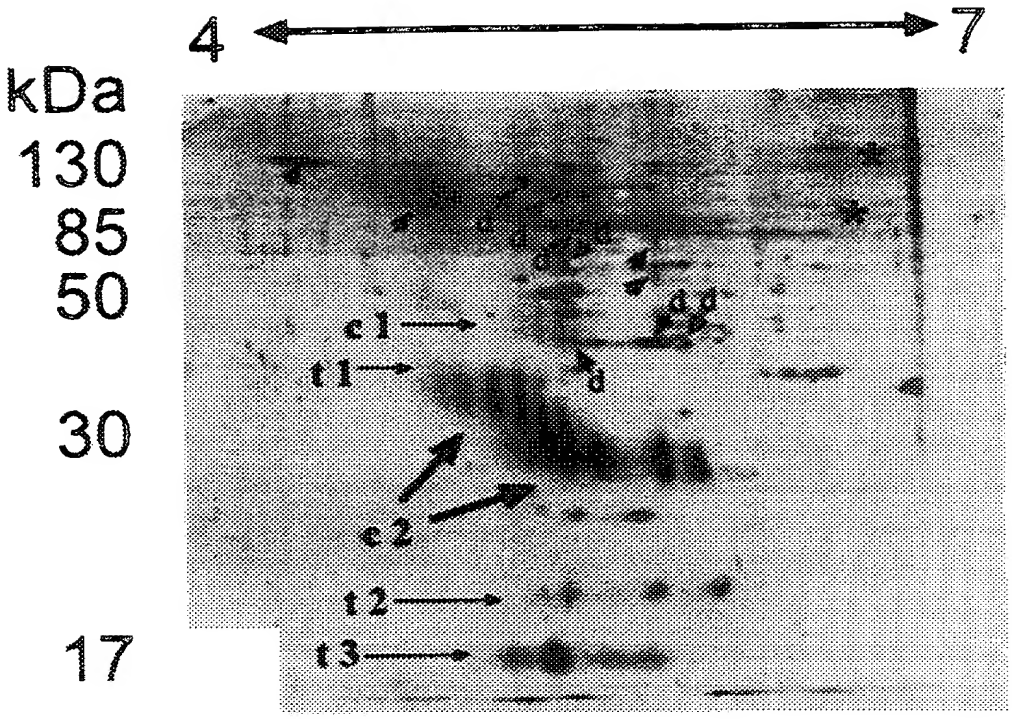


FIG.11A

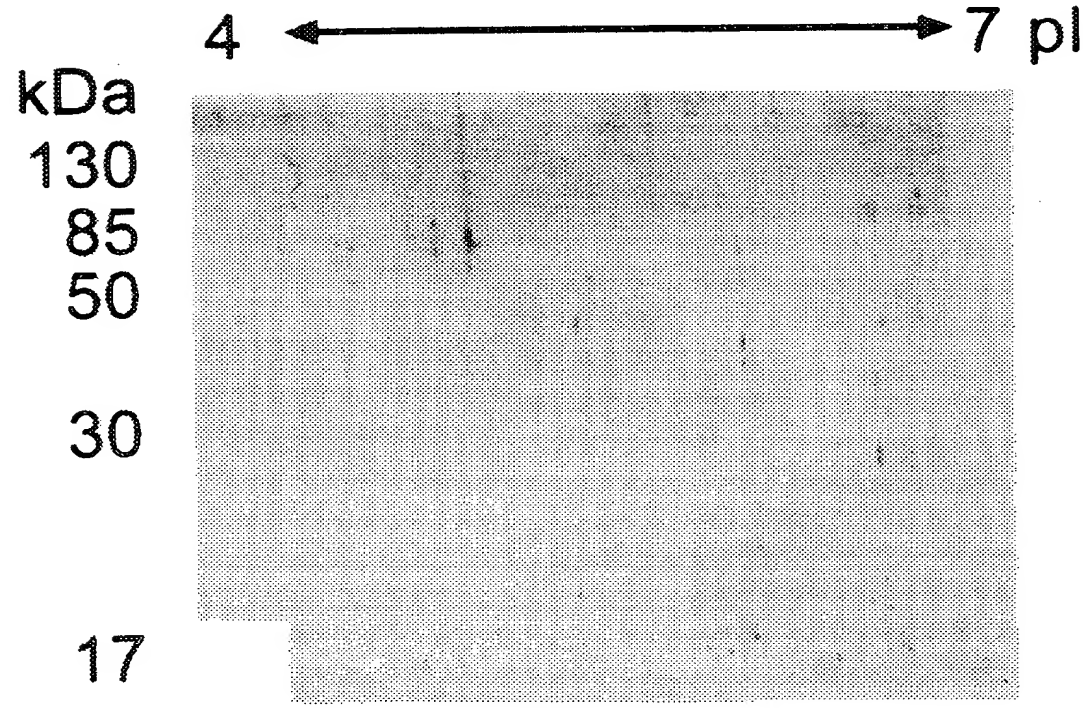


FIG.11B

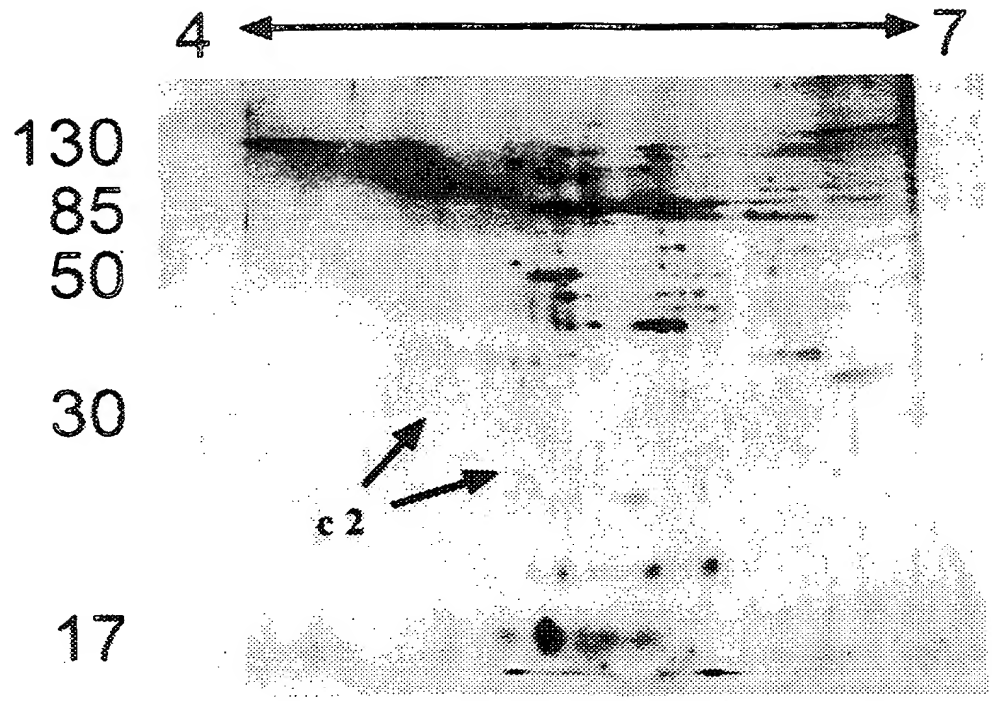


FIG. 11C

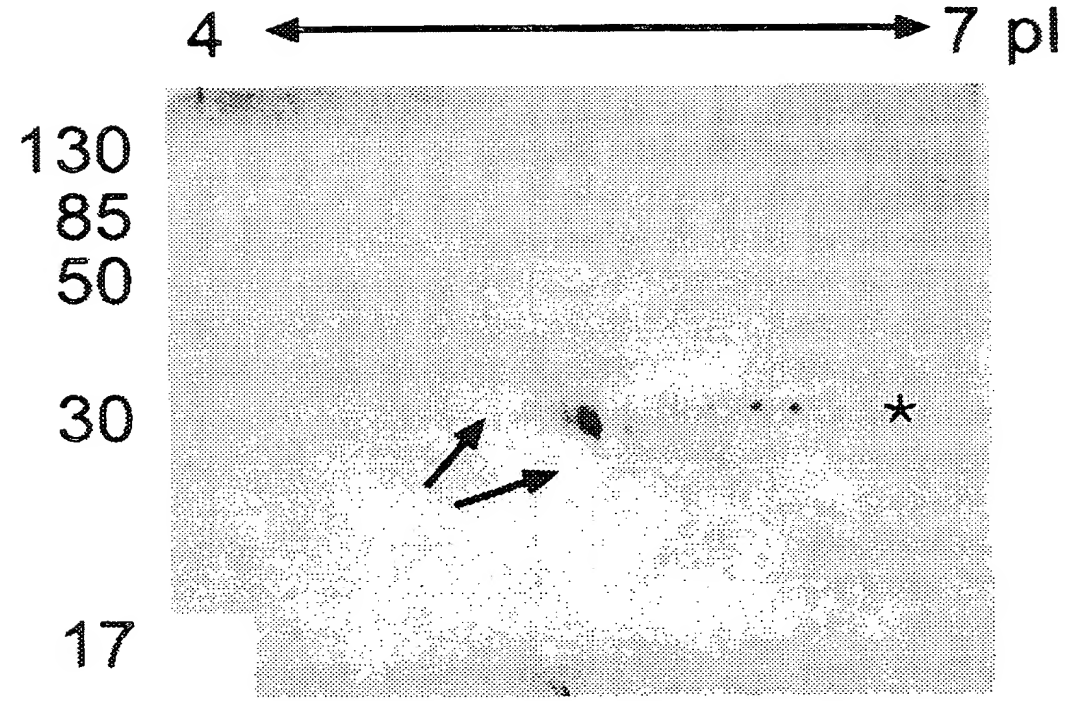


FIG. 11D

```
   1 gaggcggctg cctgctgctc tgcaggtacc atggagctga gctataggct cttcatctgc
  61 ctcctgctct ggggtagtac tgagctgtgc taccccaaac ccctctggct cttgcagggt
 121 ggagccagcc atcctgagac gtccgtacag cccgtactgg tggagtgtca ggaggccact
 181 ctgatggtca tggtcagcaa agacctttti ggcaccgggg agctcatcag ggctgctgac
 241 ctcaccttgg gcccagaggc ctgtgagcct ctggtctcca tggacacaga agatgtggtc
 301 aggttigagg ttggactcca cgagtgtggc aacagccatgc aggtaactga cgatgccctg
 361 gtgtacagca ccttcctgct ccatgacccc cgccccgtgg gaaacctgtc catcgtgagg
 421 actaaccgcg cagagattcc catcgagtgc cgctacccca ggcagggcaa tgtgagcagc
 481 caggccatcc tgcccaccig gttgcccttc aggaccacgg tgttctcaga ggagaagctg
 541 actttctctc tgcgtctgat ggaggagaac tggaacgctg agaagaggtc ccccacctic
 601 cacctgggag atgcagccca cctccaggca gaaatccaca ctggcagcca cgtgccactg
 661 cggttgtttg tggaccactg cgtggccaca ccgacaccag accagaatgc ctcccctiat
 721 cacaccatcg tggacttcca tggctgtctt gtcgacggtc tcactgatgc ctcttctgca
 781 ttcaaagttc ctcgacccgg gccagataca ctccagttca cagtggatgt cttccacttt
 841 gctaatgact ccagaaacat gatatacatc acctgccacc tgaaggtcac cctagctgag
 901 caggacccag atgaactcaa caaggcctgt tccttcagca agccttccaa cagctggttc
 961 ccagtggaag gcccggctga catctgtcaa tgctgtaaca aaggtgactg tggcactcca
1021 agccattcca ggaggcagcc tcatgtcatg agccagtggt ccacgtctgc ttcccgtaac
1081 cgcaggcatg tgacagaaga agcagatgtc accgtggggg ccactgatct tcctggacag
1141 gagtggtgac catgaagtag agcagtgggc tttgccttct gacacctcag tggtgctgct
1201 gggcgtaggc ctggctgtgg tggtgtccct gactctgact gctgttatcc tggttctcac
1261 caggagglgt cgcactgcct cccaccctgt gtctgcttcc gaataaaaga agaaa
```

FIG. 12A

```
                    MELSYRLFICLLLWGSTELCYPQPLWLLQGGASHPETSVQPVLV
ECQEATLMVMVSKDLFGTGKLIRAADLTLGPEACEPLVSMDTEDVVRFEVGLHECGNS
MQVTDDALVYSTFLLHDPRPVGNLSIVRTNRAEIPIECRYPRQGNVSSQAILPTWLPF
RTTVFSEEKLTFSLRLMEENMNAEKRSPTFHLGDAAHLQAEIHTGSHVPLRLFVDHCV
ATPTPDQNASPYHTIVDFHGCLVDGLTDASSAFKVPRPGPDTLQFTVDVFHFANDSRN
MIYITCHLKVTLAEQDPDELNKACSFSKPSNSWFPVEGPADICQCCNKGDCGTPSHSR
RQPHVMSQWSTSASRNRRHVTEEADVTVGATDLPGQEW
```

FIG. 12B

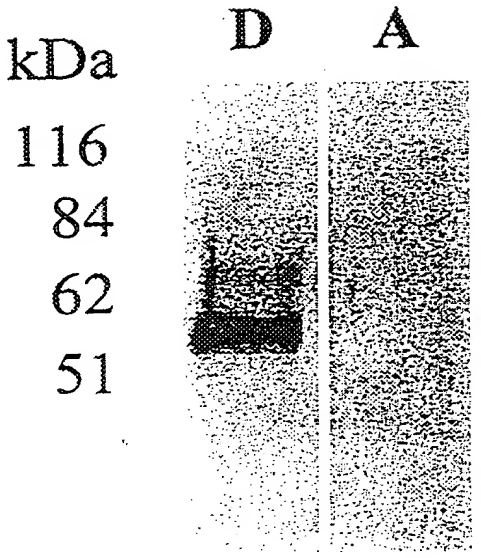


FIG.13A

D = detergent phase
A = aqueous phase

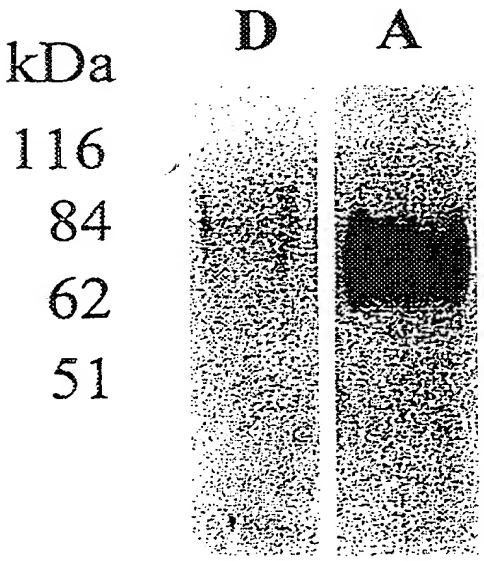
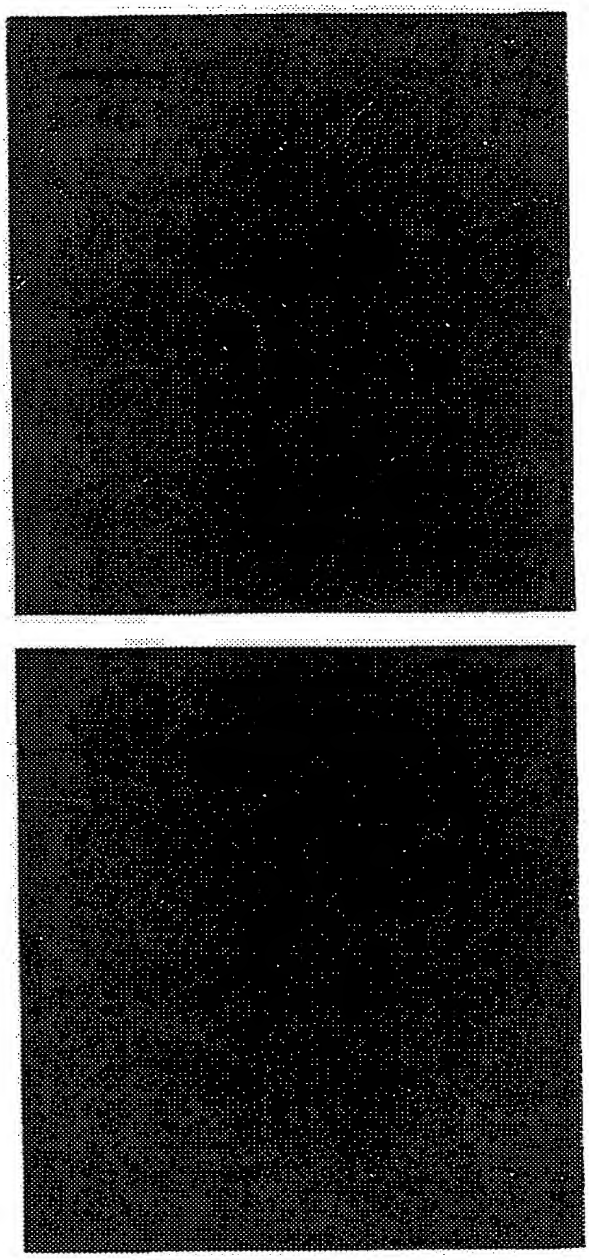


FIG.13B

D = detergent phase
A = aqueous phase



IE-10 (anti-ZP3)

Control: (rat IgG)

FIG. 14

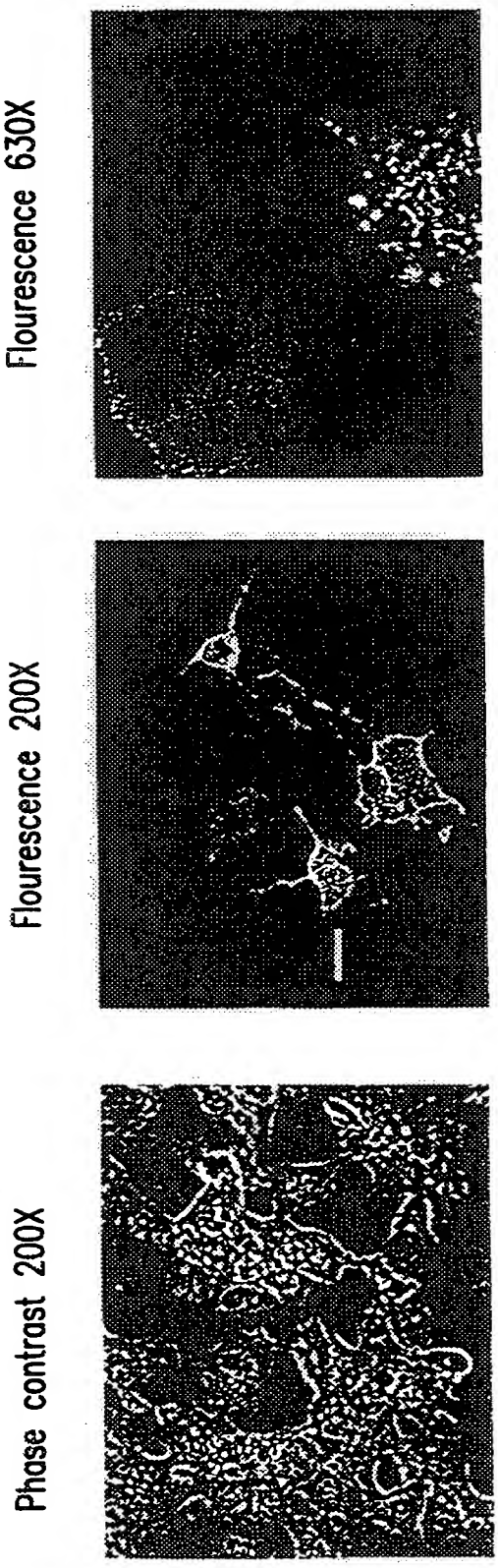


FIG. 15

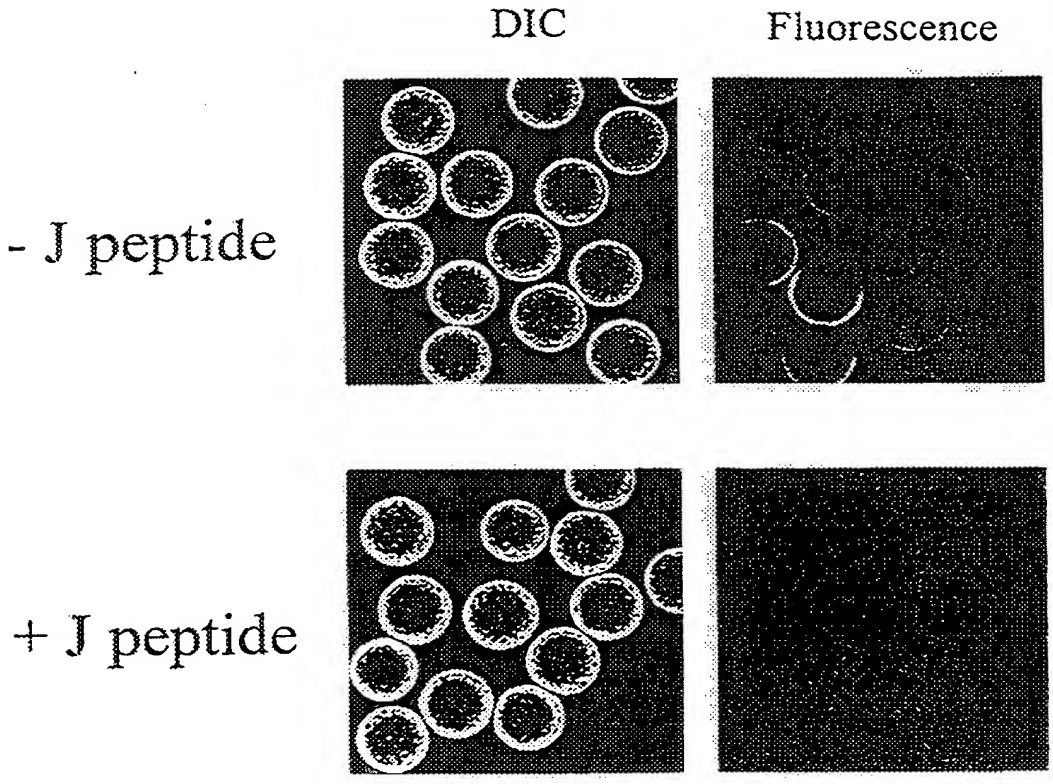


FIG. 16

EGG SURFACE PROTEINS AND METHODS OF THEIR USE FOR MODULATING FERTILITY

This application claims priority under 35 U.S.C. §119(e) to provisional patent application no. 60/089,950, filed June 19, 1998, the entire contents of which is incorporated herein by reference in its entirety. This invention was made with government support under grant numbers U54-HD29099, P30-28934, P32-DK07642, T32-HD07382, and F32-HD08002 awarded by the National Institutes of Health. The government has certain rights in the invention.

1. INTRODUCTION

The present invention is directed to egg surface antigens useful for producing antibodies which bind epitopes on the egg surface and modulate fertility. The invention encompasses compositions and methods for immunizing an individual for production of antibodies against egg surface antigens. The invention is based on the discovery of egg surface antigens involved in egg-sperm binding and fusion. Methods are provided for the use of such antigens in methods for sterilization of female animals. Methods are further provided for the use of egg surface antigens to generate antibodies useful for temporary, reversible contraception methods. Methods are further provided for the use of anti-idiotypic monoclonal antibodies which mimic egg surface epitopes to actively immunize a mammal against pregnancy.

2. BACKGROUND OF THE INVENTION

There is increasing interest in developing an immunological approach to contraception for humans and sterilization for animal populations. A contraceptive vaccine would provide many advantages over currently available methods of contraception. Methods of contraception such as hormone therapies and chemical or mechanical barriers against fertilization have serious drawbacks, such as undesirable side effects and less than complete effectiveness. For example, side effects of hormonal therapies such as the pill include cancer, and in the case of mechanical barriers, increased susceptibility to infection. In addition, contraceptive vaccines would further be useful for fertility control of animal populations, where long-term or permanent sterilization, without the need for frequent intervention, is desirable. For example, such long-term sterilization would be useful for

controlling fertility in human beings or agriculturally important livestock, such as cattle and pigs. Further, contraceptive vaccines would be useful for permanent sterilization regimes useful for pest control, such as for sterilization of rodents or other unwanted populations.

While there has been much interest in the development of immunocontraceptives, the focus has been, until recently, on the development of immunocontraceptives directed against sperm surface antigens, or on already known peptide hormones such as human chorionic gonadotropin and follicle stimulating hormone. One obstacle to the development of an effective egg surface antigen based immunocontraceptive vaccine has been the lack of knowledge regarding the molecular identities of egg surface proteins known to be directly involved in the fertilization process.

Mammalian fertilization may be defined as a series of gametic interactions in which capacitated sperm must first penetrate the cumulus cells and zona pellucida (the egg vestments), then bind to and fuse with the egg plasma membrane (oolemma). The initial binding event between gametes is known as primary binding and occurs, in the mouse model, when the zona pellucida protein, ZP3, binds to a receptor(s) on the sperm (reviewed in (McLeskey et al., 1998, Int. Rev. of Cytol. 177: 57-113). This binding event also initiates the acrosome reaction in which hydrolytic enzymes are released from the acrosomal compartment and act on the zona pellucida to facilitate penetration of the zona pellucida by sperm. Zona penetration is known as secondary binding and is mediated by the zona protein, ZP2, and one or more molecules on the inner acrosomal membrane (reviewed in Snell and White, 1996, Cell 85: 629-637).

Upon emergence from the zona pellucida, sperm then cross the perivitelline space and bind to and fuse with the oolemma. The molecular basis of sperm-oolemma binding and fusion has yet to be fully elucidated; however, recent evidence has demonstrated that integrins are involved in the interaction. Almeida et al. (1995, Cell 81: 1095-1104) found that when oocytes were treated with monoclonal antibodies against the egg surface integrin $\alpha 6\beta 1$, mouse sperm-oolemma binding was reduced. Further, these investigators reported that somatic cells which express $\alpha 6\beta 1$ bind mouse sperm avidly while somatic cells that lack $\alpha 6$ or $\beta 1$ do not. A proposed sperm surface ligand for $\alpha 6\beta 1$ is fertilin. Fertilin contains a domain homologous to a family of integrin ligands known as disintegrins (Blobel et al., 1992, Nature 356: 248-252), which suggest a cell adhesion function for the molecule. Also, recombinant fertilin is known to bind to the oolemma (Evans et al., 1997, Dev. Biol. 187:79-93), with both monoclonal antibodies to fertilin (Primakoff et al., 1987, J. Cell. Biol. 104: 141-149) and fertilin peptide analogs (Almeida et al., 1995, Cell 81: 1095-1104; Evans et al., 1995, J. Cell. Sci. 108: 3267-3278) blocking sperm-oolemma binding and fusion.

Sperm-egg binding and fusion is likely to require multiple receptor-ligand interactions and other oolemmal proteins are likely to be involved in the fertilization process. In fact, there is indirect evidence implicating other oolemmal proteins in sperm-egg interaction. A purified sperm-associated protein (protein DE) which is involved in fusion in the rat, binds to the surface of zona-free rat oocytes (Cohen et al., 1996, Biol. Reprod. 55: 200-206). Another putative oolemmal sperm receptor is removed from the surface of radioiodinated mouse eggs following trypsin treatment and reappears on the egg surface after 3-6 h of culture (Kellom et al., 1992, Mol. Reprod. Dev. 33: 46-52). The reappearance of this 94 kDa protein on the egg surface coincides with the ability of the trypsin-treated eggs to be penetrated by sperm.

Glycosyl-phosphatidylinositol (GPI)-anchored proteins may play a key role in gamete interaction. GPI-anchored proteins possess a covalently linked glycosylated phosphatidylinositol moiety which serves to attach the protein portion of the molecule to the cell surface lipid bilayer (Low and Saltiel, 1988, Science, 239: 268-275). Proteins linked to the cell surface via a phosphatidylinositol anchor are known to be involved in a wide variety of cellular functions including T cell activation, hydrolysis of extracellular matrix proteins, transduction of extracellular stimuli, and cell-cell adhesion (reviewed in Low and Saltiel, 1988, Science, 239: 268-275). GPI-anchored proteins can be released from the cell surface by treatment of cells with the highly specific enzyme phosphatidylinositol-specific phospholipase C (PI-PLC) (Low and Finean, 1978, Bioch. Biophys. Acta. 508: 565-570). Therefore, treatment of intact cells with PI-PLC has become a useful tool to characterize the released proteins and to investigate the role of GPI-anchored proteins in cell function.

Mouse sperm surface hyaluronidase (also known as PH-20) is a GPI-anchored protein and is thought to aid sperm in passage through the cumulus oopherous and possibly the zona pellucida by hydrolyzing the extracellular matrix protein, hyaluronic acid (Gmachl and Kreil, 1993, Proc. Nat. Acad. Sci. USA 90: 3569-3573); (Myles and Primakoff, 1997, Biol. Reprod., 56: 320-327). Sperm agglutination antigen-1 (SAGA-1) is another sperm surface protein which has been shown to be a GPI-linked. While its role in fertilization has yet to be elucidated, in vitro assays have demonstrated that anti-SAGA-1 monoclonal antibodies agglutinate human sperm (Diekman et al., 1997, Biol. Reprod. 57: 1136-1144).

Other reports suggest the presence of GPI-anchored proteins on oocytes. A PI-PLC sensitive GPI-linked protein, N-acetylglucosaminidase, is cleaved from the surface of Ascidians eggs following fertilization, and occupies sperm binding sites on the vitelline coat to protect the egg against polyspermy (Lambert and Goode, 1992, Dev. Biol. 154: 95-

100). Less is known about the existence of GPI-anchored proteins on the mammalian egg surface. Hirao and Yanagimachi, (1978, Gam. Res. 1: 3-12) treated hamster oocytes with a variety of enzymes including; proteases, lipases, and glycosidases and found that only phospholipase C (PLC) blocked sperm-egg fusion. Boldt et al. (1988, Biol. Reprod. 39: 19-27) treated mouse oocytes with PLC and found that the enzyme inhibited sperm-egg binding but did not inhibit sperm-egg fusion. However, due to the broad specificity of PLC for a variety of phospholipids, it has not been possible to determine if the inhibitory effects on fertilization were due to the specific release of oolemmal GPI-anchored proteins. In the only previous report which investigated the effects of PI-PLC on mammalian fertilization, Clark and Koehler (1988, Gam. Res. 19: 339-348) treated hamster oocytes with PI-PLC for 3 min and found that the enzyme had a slight, but significant, inhibitory effect on sperm-egg fusion.

The hamster oocyte is unique in that zona-free eggs from other species such as the mouse, rat, and guinea pig do not incorporate heterologous sperm as readily (Yanagimachi, 1972, J. Reprod. Fertil., 28: 477-480; Hanada and Chang, 1976, J. Reprod. Fertil., 46: 239-241; and Quinn, 1979, 210: 497-506). Because of this promiscuity, the zona-free hamster egg has been used extensively in the sperm penetration assay (SPA) to assess the fertilizing capacity of human spermatozoa (Yanagimachi et al., 1976, Biol. Reprod., 15: 471-476; Rodgers et al., 1979; Liu and Baker, 1992, Fertil. Steril., 59: 698-699). In spite of the widespread use of this assay, the molecular interactions which occur between the human sperm and hamster oocyte during gamete interaction remain largely unknown. Presumably, however, there are molecules on the hamster egg plasma membrane (oolemma) which specifically interact with molecules on the human sperm plasma membrane during sperm-egg binding and fusion.

There have been a number of recent attempts to produce contraceptive vaccines directed against egg antigens (U.S. Patent Nos. 5,820,863, 5,641,487, 5,637,300, and 4,996,297). To date, because of their relative abundance and accessibility to immunodetection, the focus has been on identifying zona pellucida epitopes. However, results from fertility trials in several species have shown that ovarian histopathology is often observed in ZP3 immunized animals. Therefore, commercial contraceptive companies have lost interest in zona proteins as contraceptive immunogens.

A new approach for an effective contraceptive vaccine that specifically targets antigens directly involved in the fertilization process is needed. However, to date, there are no reports of vaccines directed against egg protein(s) directly involved in the process of sperm-egg fusion step which is required for fertilization.

Citation or discussion of a reference herein shall not be construed as an admission that such is prior art to the present invention.

3. SUMMARY OF THE INVENTION

The present invention is directed to egg plasma membrane antigens useful for producing antibodies which will bind epitopes on the egg surface and modulate fertilization. The invention is based on the discovery that egg surface GPI-anchored proteins are directly involved in egg-sperm fusion, a key step in fertilization.

The invention further encompasses compositions and methods for immunizing an individual for production of antibodies against egg surface antigen(s). The invention is based on the discovery of a conserved set of egg surface antigens, a subset of which are released from the follicular membrane upon treatment of the membrane with PI-PLC. Methods are also provided for the use of antibodies against such antigens for active immunization, or sterilization, of female animals, by induction of a T-cell attack on the egg. These methods are particularly useful in cases where permanent sterilization is desired, for example, for sterilization of animal populations. Methods are further provided for the use of egg surface antigens to generate antibodies useful for passive immunization that can be used for reversible contraception methods. Methods are further provided for the use of anti-idiotypic monoclonal antibodies which mimic egg surface epitopes to actively immunize a mammal against pregnancy.

In another embodiment, the invention comprises a monoclonal antibody against an egg surface protein, novel hybridoma cells which express such antibodies; and methods for immunocontraception utilizing such monoclonal antibodies.

The invention further encompasses pharmaceutical compositions comprising the antigen preparation of the invention and immune-response enhancing components, together with pharmacologically acceptable carrier.

4. BRIEF DESCRIPTION OF THE FIGURES

Figure 1 (A-E) PI-PLC treatment of gametes has no effect on sperm-zona pellucida binding, however, fertilization of zona-intact oocytes is blocked. Both sperm and eggs were separately treated with 1 U per ml PI-PLC for 30 min followed by gamete co-incubation in the presence of PI-PLC. To evaluate the effects of PI-PLC on sperm-zona binding, the oocytes were washed following 1 h gamete co-incubation and prepared for observation (see methods in Example in Section 6). A single focal plane for each oocyte was selected in

which the widest diameter of the zona pellucida could be visualized and the number of sperm in that focal plane was determined. No difference in sperm-zona pellucida binding was noted between the control group treated with inactivated PI-PLC (A) and the PI-PLC treated group (B). To evaluate the effects of PI-PLC on fertilization, the oocytes were washed following 2 h gamete co-incubation and incubated overnight. Following 24 h incubation, a significant decrease in the number of fertilized oocytes (as determined by cleavage) was noted in the treatment group (D) compared to the control group (C). In Figs. 1(C) and 1(D), zygotes were treated with 1μm Hoechst # 33342 for 10 min and washed to visualize sperm which accumulated in the perivitelline space of the PI-PLC treated group (Fig. 1D, inset). Images for Figs. 1(A) and (B) (200X) were recorded using phase contrast. Images for Figs. 1(C) and (D) (200X, insets 400X) were recorded using combined phase contrast and fluorescent microscopy. Results are shown quantitatively in the histogram (E). Bars represent means +/- the standard deviation of three individual experiments. N = total number of oocytes per group. * = $p \leq 0.05$ (students T test).

Figure 2 (A-E) Treatment of sperm with PI-PLC prior to incubation with zona-free oocytes has no effect on sperm-egg binding and fusion whereas treatment of eggs with PI-PLC inhibits sperm-egg binding and fusion. Zona-free oocytes were pre-loaded with 1 μm Hoechst #33342 for 10 min and washed. Next, either sperm (A,B) or zona-free eggs (C,D) were treated with 1 U per ml PI-PLC for 30 min, washed, and the gametes were co-incubated for 40 min. The oocytes were then gently washed and prepared for observation (see methods in the Example given in Section 6). Sperm-egg binding was scored by counting the number of bound sperm per egg using phase contrast. Fusion was scored by counting the number of penetrated sperm heads using combined phase contrast and fluorescent imaging. When only sperm were treated with PI-PLC, no difference was noted in the number of sperm binding to and fusing with the egg when comparing the group treated with inactivated PI-PLC (A) to the PI-PLC treated group (B). However, when eggs were treated with PI-PLC a significant decrease in sperm-egg binding and fusion was observed when comparing the inactivated PI-PLC group (C) with the PI-PLC treated group (D). Several of the PI-PLC treated eggs had numerous bound sperm (see oocyte indicated by arrow on D) yet no fusion was recorded in most of these oocytes. Note: the fluorescent spot seen in each oocyte in (D) is the metaphase plate. Images were recorded at 200X. Results are shown quantitatively in the histogram in Fig 1(E). Bars represent means +/- the standard deviation of three individual experiments. N = total number of oocytes per group. * = $p \leq 0.05$ (students T test).

Figure 3 Treatment of oocytes with PI-PLC inhibits sperm-egg binding and fusion in a dose-dependent manner. Zona-free eggs were treated with either 5U per ml of heat inactivated PI-PLC, no PI-PLC, or increasing concentrations of PI-PLC for 30 min, washed and co-incubated with untreated sperm for 40 min. The oocytes were gently washed and prepared for observation (see methods in the Example in Section 6). Sperm-egg binding or fusion rates were not inhibited when oocytes were treated with 5U of heat inactivated PI-PLC for 30 min, however when oocytes were treated with increasing concentrations of PI-PLC, binding and fusion rates were inhibited in a dose-dependent manner, with the maximal inhibitory effect on sperm-egg binding observed at 5U/ml and fusion at 1U/ml. Each value represents the mean +/- standard deviation of three individual experiments. N = total number of oocytes per group.

Figure 4 Two-dimensional gel electrophoresis and silver staining reveals that the PI-PLC enzyme preparation is highly purified. One μg of the enzyme preparation was separated by two-dimensional electrophoresis and the gel was silver stained. Results show one prominent protein spot (~MW 30 kDa, ~pI 6) and several smaller protein spots immediately surrounding the prominent protein. See the Example in Section 6 for complete experimental details.

Figure 5 (A-B) Mouse eggs treated with PI-PLC can be artificially activated with calcium ionophore A23187. Zona-free eggs were pre-loaded with Hoechst #33342, washed, and treated with PI-PLC for 30 min. A small number of oocytes from the control group (A, inset) and treatment group (B, inset) were observed to ensure that oocytes had remained in metaphase II arrest following treatment. Oocytes were then activated with 0.5 μm calcium ionophore A23187 for 5 min, washed and cultured for 40 min. There was no observable difference in the number of eggs which resumed meiotic cell division (as determined by oocytes progressing from metaphase II arrest to anaphase II or telophase) in the group treated with inactivated PI-PLC (A) compared with the PI-PLC treated group (B). This experiment was repeated three times. Representative images were recorded using combined dual phase contrast and fluorescent microscopy (200X; inset, 100X).

Figure 6 (A-C) Treatment of zona-free oocytes with PI-PLC does not affect recognition of the α6β1 integrin by its cognate antibody. Fluorescent beads (1μM) were coated with α6β1 antibodies then incubated with zona-free eggs that were either untreated or treated with 1 U per ml PI-PLC. No difference was observed in the number of anti-α6β1 coated beads bound per egg when comparing the control group (A) with the PI-PLC treated group (B). Few

beads bound when beads were coated with an equivalent concentration of control antibody and incubated with untreated eggs (C).

Figure 7 (A-D) Two-dimensional avidin blots of biotinylated egg surface proteins demonstrate that treatment of zona-free oocytes with PI-PLC releases 70 kDa (pI 5) and 35-45 kDa (pI 5.5) protein clusters from the egg surface. (A) The repertoire of sulfo-NHS biotin labeled surface proteins extracted from eggs which were not treated with PI-PLC. The 70 kDa (pI 5) and 35-45 kDa (pI 5.5) protein clusters (indicated by arrowheads) are PI-PLC sensitive. The three protein spots denoted by asterisks bind streptavidin-HRP non-specifically. (B) No biotinylated protein spots were evident in supernatant obtained from oocytes incubated for 30 min in the absence of PI-PLC. (C) The repertoire of biotin labeled surface proteins extracted from PI-PLC treated eggs. Arrows represent the locations of 70 kDa (pI 5) and 35-45 kDa (pI 5.5) protein clusters which decline following PI-PLC treatment. (D) Supernatant from PI-PLC treated eggs reveals proteins (indicated by arrowheads) of similar molecular weights and isoelectric points to those released from the eggs surface following PI-PLC treatment. Two small protein spots at ~ 75-78 kDA and pI 5.5 (arrowheads, in D) were also released from the egg surface into the supernatant following PI-PLC treatment. These proteins were only seen in two of five replications of this experiment.

Figure 8 (A-B) Sperm-egg binding is significantly enhanced and sperm-egg fusion is not effected when human sperm are treated with PI-PLC and incubated with zona-free hamster oocytes. Human sperm were treated with either 1 U/ml PI-PLC or 1 U/ml heat inactivated PI-PLC (95°C, 5 mm) for 30 min, washed, and incubated with untreated zona-free hamster oocytes for 3 h. The oocytes were then gently pipetted to remove loosely bound sperm and the eggs were briefly incubated in acridine orange to stain chromatin. The number of sperm bound per oocyte (A) was then scored using phase contrast microscopy and sperm-egg fusion (B) was scored by counting the number of swollen sperm heads within each oocyte using fluorescent microscopy. Bars represent means +/- the standard deviation of three individual experiments. N = total number of oocytes per group * = $p < 0.05$ (students T test).

Figure 9 (A-B) Sperm-egg binding and sperm-egg fusion is blocked when zona-free hamster oocytes are treated with PI-PLC and incubated with human sperm. Zona-free hamster oocytes were treated with either 1 U/ml PI-PLC or 1 U/ml heat inactivated PI-PLC (95°C, 5 min.) for 30 min, washed and incubated with untreated human sperm for 3h. The

oocytes were then gently pipetted to remove loosely bound sperm and the eggs were briefly incubated in acridine orange to stain chromatin. The number of sperm bound per oocyte (A) was then scored using phase contrast microscopy and sperm-egg fusion (B) was scored by counting the number of swollen sperm heads within each oocyte using fluorescent microscopy. Bars represent means +/- the standard deviation of three individual experiments. N = total number of oocytes per group. *=$p \leq 0.05$ (students T test).

Figure 10 (A-B) Zona-free hamster oocytes can be artificially activated following PI-PLC treatment. To ensure that the PI-PLC preparation was not affecting oocyte viability, zona-free eggs were preloaded with Hoechst #33342, washed, and treated with either 1 U/ml PI-PLC or 1 U/ml heat inactivated PI-PLC (95°C, 5 min) for 30 min. Gocytes were then activated with 0.5 µm calcium ionophore A23 187 for 5 min, washed incubated at 37°C and 5% CO_2 for 3h. The eggs were considered activated if they had advanced from metaphase II arrest to anaphase II or telophase II (with second polar body). There was no observable difference in the number of eggs which resumed meiotic cell division in the group treated with inactivated PI-PLC (A) compared with the PI-PLC treated group (B). Arrows indicate extruded chromatin (stained blue) within the second polar body of oocytes from both groups. This experiment was repeated three times. Representative images were recorded using combined dual phase contrast and fluorescent microscopy (200X). Bar represents 20 µm.

Figure 11 (A-D) Treatment of zona-free hamster oocytes with PI-PLC releases a 25-40 kDa (pI 5-6) protein cluster from the oolemma. Zona-free hamster oocytes were biotinylated, separated into two groups (130 oocytes per group), and either mock treated or treated with 1 U/ml PI-PLC. The supernatants were collected, the eggs were washed, and the egg proteins were extracted in Celis buffer. The egg protein extracts and the proteins from the supernatants were separated by 2-D electrophoresis and electroblotted to nitrocellulose membranes. The membranes were stained with Protogold to visualize the egg proteins (red staining). The membranes were then blocked with 5% milk, probed with strepavidin-HRP, and egg surface proteins were visualized using TMB membrane peroxidase substrate (blue staining). (A) The repertoire of surface labeled oolemmal proteins from mock treated eggs. Small arrowheads indicate surface labeled proteins. Small arrowheads labeled d indicate proteins dually labeled by Protogold and TMB. Three protein trains (t1, t2, and t3) and two protein clusters (c1 and c2) were also surface labeled. The two protein spots denoted by asterisks bound strepavidin-HRP non-specifically. (B) Supernatant from mock treated oocytes. (C) The repertoire of surface labeled oolemmal proteins

following PI-PLC treatment. Arrows labeled c2 represent the location of the 25-40 kDa (pI 5-6) protein cluster which was prominent in extracts of mock treated oocytes. (D) Supernatant from PI-PLC treated oocytes. Arrows indicate a 25-40 kDa (pI 5-6) protein cluster which has a mass and isoelectric point similar to that which is released from the oocyte following PI-PLC treatment. Asterisk indicates PI-PLC isoforms.

Figure 12 (A-B) (A). Nucleotide sequence for the ZP3 human mRNA (SEQ ID NO:1). (B). ZP3 amino acid sequence (SEQ ID NO:2) as derived from the nucleotide sequence of the ZP3 human mRNA.

Figure 13 (A-B) Oolemmal ZP3 (A) partitions in the detergent phase of Triton-S 114 extracts whereas zona matrix ZP3 (B) partitions in the aqueous phase. D, detergent phase. A, aqueous phase.

Figure 14 Localization of ZP3 to the oolemma of zona-free mouse oocytes. On the left a control rat IgG antibody was used; on the right anti-ZP3 (IE-10) was used.

Figure 15 Kidney cells transfected with full length recombinant mouse ZP3 express ZP3 at the cell surface. Stained cells are shown at 200X magnification using phase contrast microscopy, and 200X and 630X using fluorescence microscopy.

Figure 16 (A-B) Specificity of the IE-10 mAb for oolemmal ZP3. Shown are without (A) or with (B) J peptide using contrast and fluorescent microscopy.

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to identification of egg surface proteins, that can be used as immunogens in a vaccine preparation to aid in the modulation of fertility. Described in detail below are the egg surface antigens, methods for their production, and methods for their use to modulate fertility.

5.1 EGG SURFACE PROTEINS AND POLYPEPTIDES

5.1.1 Egg Surface Protein Antigens

The invention relates to polypeptide antigens present on the egg plasma membrane. Such antigens have been identified as molecules directly involved in egg-sperm fusion required for fertilization. These specific glycosyl-phosphatidylinositol (GPI)-

anchored proteins are released from the egg plasma membrane by treatment of eggs with phosphatidylinositol-specific phospholipase C (PI-PLC). Release of this specific set of proteins results in a block to sperm-egg fusion and fertilization. As described in detail in the Examples set forth in Sections 6 and 7 herein below, a number of members of this class of proteins have been identified and characterized. These egg plasma membrane protein antigens are described in detail herein.

The invention is directed to mouse egg plasma membrane polypeptides, herein called M70 polypeptides, that are involved in sperm-egg fusion. The M70 polypeptides of the invention encompass polypeptides which can be isolated from mouse egg which: 1) are located on the egg plasma membrane; 2) have a molecular weight of 70kDa; 3) have a pI of 5; 4) possess a covalently linked glycosylated phosphatidylinositol moiety; and 5) are specifically released from the egg plasma membrane upon treatment with phosphatidylinositol-specific phospholipase C (PI-PLC). Other activities associated with such M70 polypeptides are antigenicity (ability to bind to an anti-M70 antibody or compete with M70 for binding), immunogenicity (ability to generate antibody which binds to M70).

In another aspect, the invention relates to polypeptides identified on the mouse egg plasma membrane, herein called M35/45 polypeptides, that are involved in sperm egg-fusion. The M35/45 polypeptides of the invention encompass polypeptides which can be isolated from mouse egg which: 1) are located on the egg plasma membrane; 2) have a molecular weight of between 35 and 45 kDa; 3) have a pI of 5.5; 4) possess a covalently linked glycosylated phosphatidylinositol moiety; and 5) are specifically released from the egg plasma membrane upon treatment with phosphatidylinositol-specific phospholipase C (PI-PLC). Other activities associated with such M35/45 polypeptides are antigenicity (ability to bind to an anti-M35/45 antibody or compete with M35/45 for binding), immunogenicity (ability to generate antibody which binds to M35/45). In various aspects of the invention the M35/45 polypeptides migrate on polyacrylamide gels as a protein with a molecular weight of 35 kDa, 36kDa, 37 kDa, 38 kDa, 39 kDa, 40 kDa, 41 kDa, 42kDa, 43 kDa, 44 kDa, and 45 kDa,.

In another aspect, the invention relates to polypeptides identified on the hamster egg surface, herein called H25/40 polypeptides, that are involved in sperm egg-fusion. The H25/40 polypeptides of the invention encompass polypeptides which can be isolated from hamster eggs which: 1) are located on the egg plasma membrane; 2) have a molecular weight of between 25 and 40 kDa; 3) have a pI of between 5 and 6; 4) possess a covalently linked glycosylated phosphatidylinositol moiety; and 5) are specifically released from the egg plasma membrane upon treatment with phosphatidylinositol-specific

phospholipase C (PI-PLC). Other activities associated with such H25/40 polypeptides are antigenicity (ability to bind to an anti-H25/40 antibody or compete with H25/40 for binding), immunogenicity (ability to generate antibody which binds to H25/40). In various aspects of the invention the H25/40 polypeptides migrate on polyacrylamide gels as a protein with a molecular weight of between 25 and 30 kDa, or between 30 and 35kDa, or between 36 and 40 kDa. In various aspects of the invention, the H25/40 polypeptides migrate on polyacrylamide gels as a protein with a molecular weight of 25 kDa, 26kDa, 27 kDa, 28 kDa, 29 kDa, 30 kDa, 31 kDa, 32kDa, 33 kDa, 34 kDa, 35 kDa, 36kDa, 37 kDa, 38 kDa, 39 kDa, and 40 kDa.

In another aspect, the invention relates to GPI-linked ZP3 polypeptide, or fragment or analog thereof. ZP3 is a well known, highly conserved, zona pellucida polypeptide. As described herein, a novel form of SP-3 has been found associated with egg membrane. This novel GPI-linked ZP3 is useful as a vaccine for sterilization or contraception of an animal or human subject. An antigenic fragment of GPI-linked ZP3 can comprise a GPI moiety linked to 1-10 amino acids, 10-30 amino acids, 30- 80 amino acids, 50-100 amino acids, 100-200 amino acids, or 200-450 amino acids of ZP3. In various aspects, the invention provides isolated M70, M35/40, and H25/40 polypeptide antigens. In other aspects, the invention provides purified M70, M35/40, and H25/40 polypeptide antigens.

In another aspect of the invention, the GPI-linked ZP3 of the invention encompasses a GPI-linked ZP3 homolog or ortholog from any species. For example, a GPI-linked egg surface protein can comprise (A) the amino acid sequence shown in Fig. 12B (SEQ ID NO:2), or, (B) an amino acid sequence encoded by a DNA sequence that hybridizes to the complement of the DNA sequence of Fig. 12A (SEQ ID NO: 1) under highly stringent conditions, *e.g.*, hybridization to filter-bound DNA in 0.5 M $NaHPO_4$, 7% sodium dodecyl sulfate (SDS), 1 mM EDTA at 65°C, and washing in 0.1xSSC/0.1% SDS at 68°C (Ausubel F.M. *et al.*, eds., 1989, Current Protocols in Molecular Biology, Vol. I, Green Publishing Associates, Inc., and John Wiley & sons, Inc., New York, at p. 2.10.3); and/or (C) an amino acid sequence encoded by DNA sequence that hybridizes to the complement of the DNA sequence of Fig. 12A (SEQ ID No: 1) under less stringent conditions, such as moderately stringent conditions, *e.g.*, washing in 0.2xSSC/0.1% SDS at 42°C (Ausubel *et al.*, 1989, *supra*), and encodes a gene product functionally equivalent to a ZP3 gene product.

The invention further relates to fragments (and derivatives and analogs thereof) of M70, M35/45, H25/40 and GP1-linked ZP3, which comprise one or more domains of an M70, M35/45, or H25/40 protein. In one aspect the M70, M35/45, or

H25/40 fragment is a glycolsylated phosphotidyinositol linked fragment. In another aspect the M70, M35/45, or H25/40 fragment is an extracellular domain fragment.

In one aspect of the invention, egg plasma membrane polypeptides, such as M70, M35/45, H25/40, and GP1-linked ZP3, bind to a sperm protein to facilitate sperm-egg fusion.

Egg plasma membrane polypeptides involved in sperm-egg fusion, such as M70, M35/45, H25/40, and GP1-linked ZP3, or peptide fragments thereof, can be prepared for a variety of uses. For example, such polypeptides, or peptide fragments thereof, can be used for the generation of antibodies, for use in diagnostic and therapeutic assays, or for the identification of agents or small molecules that modulate sperm-egg fusion process required for fertilization.

5.2 PURIFICATION OF EGG SURFACE PROTEINS

Egg plasma membrane polypeptides involved in sperm-egg fusion, such as M70, M35/45, H25/40, and GP1-linked ZP3, can be purified from any cell type that expresses such polypeptides, such as mouse or hamster egg cells. In a preferred embodiment, the M70, M35/40, and H25/40 polypeptides are purified from a fraction of an extract of such cells enriched for cell membrane components. For example, egg plasma membrane polypeptides, such as M70, M35/40, and H25/40 proteins can be solubilized from cells, or cell extracts, such as a cell membrane fraction. The solubilized proteins can subsequently be purified by various procedures known in the art, including but not limited to chromatography (*e.g.*, ion exchange, affinity, and sizing chromatography), centrifugation, electrophoretic procedures, differential solubility, or by any other standard technique for the purification of proteins.

As exemplified by the Examples set forth in Sections 6 and 7, protein "spots" that appear in samples from normal eggs, but are absent in PI-PLC treated eggs can be analyzed further. Differences can be detected by visual inspection of gels, or by using densitometry and computerized image analysis thereby facilitating spot detection, background subtraction and spot matching (*see* Pennington et al., 1997, Trends Cell Biol. 7: 168-73). Further, egg plasma membrane polypeptides, such as M70, M35/40, or H25/40 protein can be detected by Western Blot analysis of 2D gels, if antibody is available (Harlow and Lane, 1988, *supra*). Once identified, the molecular weight (MW) and the isoelectric point (pI) of an egg surface protein can be determined by calibrating its position relative to known standards run in parallel on 2D gels. Specific proteins can then be purified, and their sequence determined, or a portion of their sequence determined, by

techniques well known in the art, such as Edman degradation sequencing (Edman and Begg, 1967, Eur. J. Biochem. 1:80-91; see, *e.g.*, Creighton, 1983, "Proteins: Structures and Molecular Principles," W.H. Freeman & Co., N.Y., pp.34-49), automated by electroblotting onto polyvinylidene difluoride (PVDF) membranes using Edman degradation chemistry determined by gas-liquid phase, liquid-pulse or solid phase sequence analysis (Findlay and Geisow, 1989, Protein Sequencing: A Practical Approach, IRL Press, Oxford, pp. 1-199). Alternatively, proteins and peptides can be characterized by mass spectrometry, using peptide-mass fingerprinting or protein sequencing methodologies to identify sequence information and post-translational modifications (Dainese et al., 1997, Electrophoresis, 18:432-42; Mann and Wilm, 1995, Trends Biochem. Sci., 20:219-24; Yates, 1996, Methods Enzymol. 271:351-77). After limited sequence information is obtained, protein (Swiss-Prot; http://www.expasy.ch) and nucleic acid sequence (Genebank and EMBL; http://ncbi.nlm.nih.gov) databases can be searched to determine if the protein sequence is novel. Proteins will be analyzed further, used to generate antibodies, as described in Section 5.7, and used for identification of nucleic acid sequences. The amino acid sequence obtained may be used as a guide for the generation of oligonucleotide mixtures that can be used to screen for gene sequences encoding such proteins.

The egg plasma membrane proteins of the present invention can be analyzed by assays based on their physical, immunological, or functional properties. The amino acid sequences of egg plasma membrane polypeptides involved in sperm-egg fusion, such as M70, M35/45, H25/40, and GP1-linked ZP3, can be derived by deduction from the DNA sequence if such is available, or alternatively, by direct sequencing of the protein, *e.g.*, with an automated amino acid sequencer. The protein sequences can be further characterized by a hydrophilicity analysis (Hopp and Woods, 1981, Proc. Natl. Acad. Sci. U.S.A. 78:3824-3828). A hydrophilicity profile can be used to identify the hydrophobic and hydrophilic regions of the protein (and the corresponding regions of the gene sequence, if available, which encode such regions).

Secondary structural analysis (Chou and Fasman, 1974, Biochemistry 13:222) can also be done, to identify regions of the egg surface protein sequence that assume specific secondary structures. Other methods of structural analysis can also be employed. These include but are not limited to X-ray crystallography (Engstom, 1974, Biochem. Exp. Biol. 11:7-13) and computer modeling (Fletterick, R. and Zoller, M. (eds.), 1986, Computer Graphics and Molecular Modeling, in Current Communications in Molecular Biology, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York).

Alternatively, egg plasma membrane polypeptides involved in sperm-egg fusion, such as M70, M35/40, and H25/40 may be isolated and/or purified using

immunological procedures. In one embodiment, where an antibody specific to M70, M35/40, or H25/40 is available, a method of the invention for isolating M70, M35/40, or H25/40 protein comprises the steps of: a) preparing an extract of egg cells; b) contacting a M70, M35/40, or H25/40 specific antibody with the extract for a time period sufficient for the M70, M35/40, or H25/40 in the extract to bind the antibody; and c) recovering the bound antibody. In another embodiment, the method can also be used with an antibody that comprises an affinity tag. Accordingly, the method further comprises incubating the extract and the tagged antibody to the M70, M35/40, or H25/40 protein with a solid phase surface containing a binding partner of the affinity tag for a time period sufficient to allow binding of the egg plasma membrane polypeptides, such as M70, M35/40, or H25/40 to the solid phase surface prior to the recovery step. The isolated protein can then be eluted from the antibody.

Egg plasma membrane polypeptides, such as M70, M35/40, and H25/40 can be purified by isolating cell membranes and purifying the egg plasma membrane polypeptides from other membrane components. Membranes can be isolated from the egg cells of the invention, prepared according to the methods described in Section 6, below. Cells can be lysed and the plasma membrane fraction can be isolated from cells using procedures known in the art, such as dextran/polyethylene glycol biphase separation. Plasma membranes can be treated with a buffer which dissociates membrane-associated proteins from the lipid bilayer (*e.g.*, a buffer containing a non-ionic detergent such as Nonidet P-40™, Triton X-100™, or sodium deoxycholate). Proteins can be purified away from membrane lipids using conventional dialysis procedures.

In one embodiment, the crude dialysed protein preparation can be applied to an affinity column composed of antibody or antiserum stabilized on an appropriate matrix. Membrane-associated proteins other than M70, M35/40, or H25/40 will pass through the column, while M70, M35/40, or H25/40 will remain bound. Non-specific binding of other membrane components can be reduced by increasing the salt concentration and varying the pH of the buffer in which the crude protein preparation is dissolved. Thorough washing of the column after application of the crude protein preparation can further reduce binding of non-specific proteins.

In another embodiment, egg plasma membrane polypeptides, such as M70, M35/40, or H25/40 protein may be purified using specific antibodies, previously generated against M70, M35/40, or H25/40 protein (see Section 5.7, *infra*), or cell membranes. The crude protein preparation from membrane dialysis is applied to an antibody affinity column which is composed of M70, M35/40, or H25/40-specific antibody, stabilized on an appropriate matrix. Antibody-coupled resin, or filter methods can also be used, or other

antibody affinity techniques known in the art (see, for *e.g.*, Harlow and Lane, 1988, *supra*). The column or resin can be washed with buffer to remove proteins which bind non-specifically. The protein which remains bound to the column is eluted by conventional procedure such as washing with a buffer containing high salt or low pH.

In yet another embodiment, egg plasma membrane polypeptides, such as M70, M35/40, or H25/40 protein may be purified by sizing or ion exchange column chromatography. FPLC may be used to facilitate purification of large amounts of protein. If antibody to M70, M35/40, or H25/40 is generated or is available, protein may be detected and followed during purification by Western blot or ELISA (enzyme-linked immunosorbent assay) analysis.

5.3 ISOLATION OF NUCLEIC ACID SEQUENCES

The egg plasma membrane polypeptide nucleotide sequences of the invention can be isolated directly from mRNA, cDNA or from a cDNA or genomic library. Alternatively, egg plasma membrane polypeptide cDNA can be isolated indirectly by first isolating and characterizing the egg plasma membrane polypeptide protein, and subsequently using the egg plasma membrane polypeptide sequence to identify gene sequences in a cDNA or genomic library. Details of such methods are fully described herein.

Nucleic acids encoding egg plasma membrane polypeptides involved in sperm-egg fusion, such as M70, M35/45, H25/40, and GP1-linked ZP3, can be isolated by a variety of methods well known to those of skill in the art, including, but not limited to: screening a cDNA expression library in mammalian cells using egg plasma membrane polypeptide specific antibodies, or "panning"; screening a cDNA expression library in bacterial cells; or differential expression methods such as screening a subtracted cDNA library with an egg plasma membrane polypeptide nucleic acid probe or a specific antibody.

5.3.1 Preparation of mRNA and cDNA

The methods for the purification of mRNA and the synthesis of complementary DNA (cDNA) from egg RNA are described herein. The procedures described in standard treatises, *e.g.*, Methods in Enzymology, 1987, volume 154, Academic Press; Sambrook *et al.*, 1989, Molecular Cloning - A Laboratory Manual, 2nd Edition, Cold Spring Harbor Press, New York; and Ausubel *et al.*, eds., 1992, Current Protocols in Molecular Biology, Greene Publishing Associates and Wiley Interscience, New York, may be followed to carry out routine molecular biology reactions in purification of mRNA.

Methods described in detail *infra* are for illustration only and not by way of limitation. Various mRNA and cDNA preparation systems that are commercially available may also be used according to the manufacturer's instructions for making the mRNA and cDNA of the invention.

Total ribonucleic acid (RNA) may be isolated from egg cells by a variety of methods known in the art depending on the source and amount of cells. It is preferable to obtain good quality RNA that is of high molecular weight in order to construct cDNA libraries that contain even rarely expressed gene products. To prepare high quality RNA, methods that provide complete lysis of cells, and rapid inactivation of nucleases are preferred. A single-step RNA preparation method uses the strong chaotropic agent, guanidinium isothiocyanate, with a mild detergent and 2-mercaptoethanol or dithiothreitol to denature proteins and inactivate nucleases, followed by purification of the RNA by ultracentrifugation (Chomczynski and Sacchi, 1987, Anal Biochem 162:156-159; Chomczynski, 1989, U.S. Patent No. 4,843,155). This procedure may also be used especially when isolating RNA from small quantities of cellular material.

Preferably, total RNA isolated from cells is further purified before conversion into complementary DNA (cDNA). Since the vast majority of eukaryotic messenger RNA (mRNA) molecules contain tracts of poly(adenylic) acid (poly-A) at the 3′ end, it can be enriched by affinity chromatography using oligo-dT cellulose (Aviv and Leder, 1972, Proc. Natl. Acad. Sci., 69:1408-1412). Total RNA is denatured to expose the poly-A tails. Poly-A+ RNA is then bound to oligo-dT cellulose, with the remainder of the RNA washing through. The poly-A+ RNA is eluted by removing salt from the solution. This step may be repeated to further enrich for messenger RNA. A wide variety of oligo-dT matrices in different configurations may also be used, including but not limited to, simple gravity columns, para-magnetic particles, and spin columns. Substituted oligo-dT, such as biotinylated oligo-dT, may also be used. The quantity and quality of RNA thus obtained may be determined by methods such as formaldehyde agarose gel electrophoresis. The use of RNA enriched for poly-A+ RNA is most preferred.

Conversion of RNA into double-stranded cDNA can be accomplished by a number of different procedures well known in the art. See for example, Okayama and Berg, 1982, Mol. Cell Biol. 2:161-170; Gubler and Hoffman, 1983, Gene 25:263-269; and Huse and Hansen, 1988, Strategies (Stratagene) 1:1-3. The first step in the making of cDNA involves the oligonucleotide-primed synthesis of a first strand cDNA by reverse transcriptase. For example, mRNA hybridized to an oligo-dT primer can be copied into DNA by a reverse transcriptase, such as AMV reverse transcriptase, MMLV reverse transcriptase, or Superscript (Kotewicz *et al.*, 1988, Nucleic Acid Res. 16:265-277).

Random hexamers may be used to prime first-strand synthesis from internal sites within the mRNA instead of oligo-dT primers resulting in shorter cDNAs which are enriched for the 5' ends of long messenger RNAs.

The next step in the process involves synthesizing the second strand cDNA and producing suitable DNA ends for insertion in a cloning vector. Briefly, for example, the second strand cDNA may be synthesized using E. coli DNA polymerase I, Klenow fragment using the RNA-DNA as a template. The RNA in the RNA-DNA hybrid can be removed with RNase H, and gaps in the newly synthesized second strand cDNA can be filled in by E. coli DNA polymerase I. The fragments of second strand cDNAs thus produced are ligated with E. coli DNA ligase to form a contiguous second strand cDNA.

After second strand DNA synthesis, the double stranded cDNA requires further repair with enzymes, such as RNase H, RNase A, T4 DNA polymerase and E. coli DNA ligase, to form perfectly matched strands (*i.e.*, having "flush" or "blunt" ends).

In some protocols, where the amount of starting cellular material is very limited, the cDNA can be amplified *in vitro*, by nucleic acid amplification methods known in the art, such as polymerase chain reaction (PCR) and ligation-mediated chain reaction (LCR). Generally, first strand oligo-dT primed cDNA obtained by a standard method is extended with a oligo-dG tail by terminal transferase, and a second primer containing a oligo-dC segment is used to prime second strand synthesis with a thermostable DNA polymerase. This procedure produces a double-stranded cDNA population each molecule of which is bracketed by two oligonucleotides of known sequence. Using the appropriate set of primers, standard PCR can be used to amplify the cDNA. See, for example, U.S. Patent Nos. 4,683,202, 4,683,195 and 4,889,818; Gyllenstein *et al.*, 1988, Proc. Natl. Acad. Sci. USA 85:7652-7656; Ochman *et al.*, 1988, Genetics 120:621-623; Loh *et al.*, 1989, Science 243:217-220; Tam *et al.*, 1989, Nucleic Acid Res. 17:1269; Belyavsky *et al.*, 1989, Nucleic Acid Res. 17:2919-2932. In specific embodiments of the invention, RT-PCR can be used to generate amplified cDNAs from the RNAs (see, *e.g.*, Domec *et al.*, 1990, Anal Biochem, 188:422-426; Van Gelder *et al.*, 1990, Proc. Natl. Acad. Sci., 87:1663-1667).

In another embodiment, the egg plasma membrane polypeptide nucleic acid sequences can be isolated by identifying genes that are expressed in eggs but not in other cell types (such as sperm or somatic cells). A number of methods exist for identifying such differentially expressed genes between two or more cell types. For example, differential display of cDNA 3' end sequences (Liang and Pardee, 1992, Science 257:967-971), serial analysis of gene expression by comparative gels of PCR products (SAGE; Velculescu et al., 1995, Science 270:484-487), or nucleic acid array (DNA chip) technology (Schena et al.,

1995, Science 270:467-470; see also, J. Ramsey, 1998, Nat. Biotechnology 1:40-44), can be used to identify differentially expressed genes in a non-selective manner.

For example, differential display can be used identify egg plasma membrane polypeptide cDNA sequences present in egg cells but absent in control cells. Egg cells and negative control cells are prepared, and mRNA is isolated as described, *supra*. cDNA is prepared from mRNA, as described, *supra*, using RT-polymerase chain amplification with a set of labeled oligonucleotide primers designed to identify the 3' ends of mRNAs (Liang and Pardee, 1992, *supra*). Primers used for the synthesis of the first strand each contain a stretch of oligo dT at its 5' end, followed by a pair of nucleotides at its 3' end. Such oligonucleotides are end-labeled and used as primers in reverse-transcriptase polymerase chain reactions (RT-PCR) to generate a population of specific cDNAs. Products of such RT-PCR reactions are displayed on a sequencing gel. Using mRNAs derived from different populations of cells, the pattern of displayed products can be compared to identify bands that are unique to different cell types (Liang and Pardee, 1995, Curr. Opin. Immunol., 7:274-280; McClelland, M. et al., 1995, Trends Genet., 11, 242-246).

mRNA from eggs is compared to mRNA from control cells by differential display. Following RT-PCR using the specific set of primers described hereinabove, RT-PCR products are displayed on thin polyacrylamide gels containing 8% urea, the type used for DNA sequencing analysis. Products that are detected in egg cells but absent in control cells are chosen to be analyzed further. Gel purification and sequence analysis of such products can be performed to identify egg plasma membrane polypeptide nucleic acid candidates. Protein-coding sequences of egg plasma membrane polypeptide candidates, i.e., sequences present in egg cells but not in control cells, can be compared to known protein sequences in a data base such as Swiss-prot (Bairoch and Apweiler, 1998, Nucl. Acids Res. 26:38-42; http://www.expasy.ch). Novel sequences can be chosen as potential egg plasma membrane polypeptide candidates. Such gene products can then be isolated from the cDNA population using standard cloning techniques (Ausubel *et al.*, eds., 1992, Current Protocols in Molecular Biology, Greene Publishing Associates and Wiley Interscience, New York), and can be tested for their ability to bind egg plasma membrane polypeptide antibodies.

In another embodiment, nucleic acid array technology can be used to identify egg plasma membrane protein-specific sequences. Such micro-arrays of cDNA probes have been successfully used to compare the expression patterns of different cell types (DeRisi, *et al.*, 1996, Nat. Genet.14:457-460). Micro-arrays typically have many different DNA molecules fixed at defined "addresses" on a two dimensional, usually glass, support. Each address contains either many copies of a single DNA, or a mixture of different DNA molecules, and each DNA molecule is usually 1000 nucleotides or less in length. The

DNAs can be from any source, cDNA libraries, or can be synthesized oligonucleotides. A vast excess of probe is fixed at each address, so that the hybridization signal intensity at that address is limited by the concentration of labeled complementary sequence in immediate proximity to the address. The probe array is useful for measuring the ratio of hybridization between to differently labeled samples that are thoroughly mixed and therefore share the same hybridization conditions. Simple probe arrays are currently able to detect cDNA species that are present at 2 to 10 copies of mRNA per cell when contacted with a solution containing a total cDNA concentration of 1 mg/ml. In a preferred embodiment, mRNA derived from egg plasma membrane polypeptide positive hybrid cells and control cells is labeled with distinct fluorophores and hybridized to DNA on a micro-array in a mixture. The sequences of differentially expressed nucleic acids are determined by identifying the addresses where differential hybridization between the two cell populations occurs. These nucleic acid sequences can then be used to identify egg plasma membrane polypeptide gene sequences, to synthesize recombinant protein, and to generate antibodies.

cDNAs can be inserted into an appropriate cloning vector, and introduced into an appropriate host organism for propagation. Such cDNA libraries may then be used for preparation of "subtracted" cDNA libraries, or for direct and expression screening for egg plasma membrane polypeptide gene sequences. Methods for such procedures are well known in the art, and are described in standard treatises, *e.g.*, Methods in Enzymology, 1987, volume 154, Academic Press; Sambrook *et al.*, 1989, Molecular Cloning - A Laboratory Manual, 2nd Edition, Cold Spring Harbor Press, New York; and Ausubel *et al.*, eds., 1992, *supra*.

Selective protocols can be used to specifically increase the abundance of sequences overexpressed in one population relative to another by elimination of gene products common for both from the two populations by means of subtractive hybridization. A number of such subtraction hybridization protocols can be used, including, but not limited to, representational difference analysis (Fargnoli et al., 1990, Anal. Biochem., 187:364-73; Wang and Brown, 1991, Proc. Natl. Acad. Sci. 88:11505-09; see Lisitsyn, 1995, Trends Genet. 11:303-7), enzymatic degrading subtraction (EDS; Zeng *et al.*, 1994, Nuc. Acid Res. 22:4381-85), RecA-mediated subtraction hybridization (Hakvoort *et al.*, 1996, Nucl. Acids Res. 24:3478-80) or selective amplification via biotin and restriction mediated enrichment (SABRE; Lavery *et al.*, 1997, Proc. Natl. Acad. Sci. USA 13:6831-36). Briefly, a subtracted library is prepared from eggs and negative control cells. The egg cells and control cells are prepared, and egg cDNA is hybridized to a large excess of poly A+ mRNA from control cells. cDNA molecules expressed only in eggs will not hybridize, and can be removed by passing mixture over a hydroxylapatite column under conditions

such that the column specifically retains RNA:DNA duplexes but not DNA or DNA duplexes. The column flow-through, containing cDNAs representing mRNAs that are expressed in eggs but not in negative control cells, is cloned into an appropriate vector.

In another embodiment, biotinylated RNA or cDNA can be used to label negative control cells. After hybridization, the egg specific sequences can be eliminated by passing the hybridization mixture over a streptavidin column. The flow-through can be cloned into an appropriate expression vector. In another embodiment, any of the above mentioned techniques for subtractive hybridization techniques can be used, or any of the other techniques known in the art. The library thus created is plated out to isolate single colonies. In one embodiment, since this cDNA library contains egg-specific sequences that are absent in negative control cells, the cDNA inserts can simply be sequenced at this point. In another embodiment, the colonies can be transferred to nitrocellulose filters. The filters can be incubated with antibody specific to egg plasma membrane polypeptide, and the antibody detected using methods known in the art. In yet another embodiment, the colonies can be transferred to nitrocellulose filters, and incubated, under conditions that promote nucleic acid hybridization, with a nucleic acid probe. For example, if egg plasma membrane polypeptide sequence information is available, a labeled degenerate oligonucleotide can be designed. Protein sequence information is used to design degenerate oligonucleotides containing all possible codons for egg plasma membrane polypeptide amino acids. Sequence information from various regions of the protein can be used to generate a series of such degenerate pools of oligonucleotides, where each oligonucleotide pool contains some sequences that are complementary to egg plasma membrane polypeptide gene sequences. Such degenerate oligonucleotide pools can be used to screen the subtracted cDNA library, cDNA inserts can be sequenced, and sequence of positive clones can be used to screen a genomic library and thus identify egg plasma membrane polypeptide genes.

The cDNA inserts can be sequenced, and the sequence of positive clones can be used to screen a genomic library and thus identify egg plasma membrane polypeptide gene.

The present invention provides various methods for isolation of nucleic acid molecules encoding egg plasma membrane polypeptide by screening cDNA and/or genomic DNA library. A gene library comprises a pool of nucleic acid molecules, in which one or more nucleic acid molecules comprise nucleotide sequences encoding egg plasma membrane polypeptide or a fragment thereof. A gene library can be introduced into the appropriate recombinant cells for replication and screening and for production of the proteins encoded by the cDNAs.

In one embodiment, the invention provides a method for screening a gene library for the egg plasma membrane polypeptide gene using one or more nucleic acid probe, such as a pool of degenerate oligonucleotides having sequences that encode egg plasma membrane polypeptide or a fragment thereof. The nucleic acid sequence of the probe can be designed in accordance to available peptide sequence of an egg plasma membrane polypeptide, a fragment or homolog thereof. For example, a probe based on the egg plasma membrane polypeptide peptide sequence of one species can be used to identify and isolate the egg plasma membrane polypeptide gene of a related species. Egg plasma membrane polypeptide can be purified and sequenced. Protein sequence information is then used to design degenerate oligonucleotides containing all possible codons for egg plasma membrane polypeptide amino acids. Sequence information from various regions of the protein can be used to generate a series of such degenerate pools of oligonucleotides. Thus, each oligonucleotide pool contains some sequences that are complementary in its entirety to egg plasma membrane polypeptide gene sequences. Such degenerate oligonucleotide pools can be used to screen a gene library, prepared as described herein, supra. Accordingly, the method comprises (a) incubating a labeled nucleic acid probe with DNA molecules derived from recombinant cells containing a plurality of DNA molecules from egg plasma membrane polypeptide positive hybrid cells, for a time period sufficient to allow hybridization of the labeled probe to the DNA molecules, wherein the labeled probe having a nucleic acid sequence that comprises a sequence that encodes egg plasma membrane polypeptide or a fragment thereof; (b) identifying the recombinant cell containing the DNA molecule to which the labeled probe bound; (c) recovering the DNA molecule present in the recombinant cell.

In another embodiment, the invention provides methods for identifying and isolating the egg plasma membrane polypeptide gene that rely on expression of cDNA insert and screening for its activity by binding assays, immunological methods, or an altered cellular phenotype. The egg plasma membrane polypeptide cDNA can be isolated indirectly by screening the cDNA expression library for egg plasma membrane polypeptide activity, such as egg plasma membrane polypeptide antibody-binding activity. For example, egg plasma membrane polypeptide antibodies can be labeled with a detectable compound, such as a radioactive, fluorescent or biotinylated compound, and used as probes to screen bacterial colonies that have been induced to express cDNA inserts.

A eukaryotic expression library can be screened by "panning" (Seed, 1987, Proc. Natl. Acad. Sci. USA 84:3365-69). This method is particularly preferred for screening cDNA molecules encoding proteins that are expressed on the cell surface. Using this technique, culture dishes are pre-coated with antibody, which can bind to cells that

express egg plasma membrane polypeptide. Alternatively, culture dishes may be coated with an egg plasma membrane polypeptide ligand, which also can bind to cells that express egg plasma membrane polypeptide. Non-adherent cells can be rinsed away, and selected cells can be isolated and their inserts can be further analysed. It is preferable that the type of host cell used in panning is non-adherent to surfaces of cell culture containers, such as plastic, so as to facilitate the screening methods of the invention. In one embodiment, an SV40 vector and control sequences are utilized, and the resulting cDNA library is introduced into African green monkey cells (COS cells). The cDNA library can be constructed in a vector containing viral control regions, and introduced in mammalian cells by transfection or infection with viral vectors. Cells are distributed on microtiter dishes for screening. The cDNA library can be transiently expressed in mammalian cells. In a preferred embodiment, the cDNA used in constructing the library is prepared from mRNA isolated from the egg plasma membrane polypeptide positive hybrid cells of the invention. In another embodiment, the library is a subtracted cDNA library, wherein gene products common to both egg plasma membrane polypeptide positive hybrid cells and egg plasma membrane polypeptide negative hybrid cells are eliminated from the egg plasma membrane polypeptide positive hybrid cell mRNA or cDNA population by means of subtractive hybridization prior to construction of the cDNA library. In yet another embodiment, the library is an cDNA library, or a "subtracted" cDNA library, in which cDNAs common to both egg and sperm, or a somatic cell, are subtracted from egg cDNA population prior to cloning (Fargnoli et al., 1990, Anal. Biochem., 187:364-73; Wang and Brown, 1991, Proc. Natl. Acad. Sci. 88:11505-09; Lisitsyn, 1995, Trends Genet. 11:303-7).

An expression construct, as used herein, refers to a polynucleotide comprising egg plasma membrane polypeptide positive hybrid cell derived cDNA sequences operably associated with one or more regulatory regions which enables expression of the library of cDNAs in an appropriate host cell. "Operably-associated" refers to an association in which the regulatory regions and the cDNA sequence to be expressed are joined and positioned in such a way as to permit transcription, and ultimately, translation.

The regulatory regions necessary for transcription of the cDNA library can be provided by an expression construct. A translation initiation codon (ATG) may also be provided if the cDNA fragments without their cognate initiation codon are to be expressed. In a compatible host-construct system, cellular transcriptional factors, such as RNA polymerase, will bind to the regulatory regions on the expression construct to effect transcription of the cDNA library in the host organism. The precise nature of the regulatory regions needed for gene expression may vary from host cell to host cell. Generally, a

promoter is required which is capable of binding RNA polymerase and promoting the transcription of an operably-associated nucleic acid sequence. Such regulatory regions may include those 5'-non-coding sequences involved with initiation of transcription and translation, such as the TATA box, capping sequence, CAAT sequence, and the like. The non-coding region 3' to the coding sequence may contain transcriptional termination regulatory sequences, such as terminators and polyadenylation sites. In order to be "operably-associated", it is not necessary that the regulatory region and the cDNA sequences be immediately adjacent to one another. Regulatory regions suitable for gene expression are well known in the art (see Section 5.6).

Both constitutive and inducible regulatory regions may be used for cDNA expression. It may be desirable to use inducible promoters when the conditions optimal for growth of the host cells and the conditions for high level expression of the cDNA library are different. This use of an inducible regulatory region may be particularly desirable if some of the proteins encoded by the cDNAs confer growth advantages or disadvantage to the recombinant host cells expressing them. Examples of useful regulatory regions are provided in the next section below.

The expression constructs comprising the cDNA library operably associated with regulatory regions can be directly introduced into appropriate host cells. See, for example, U.S. Patent No. 5,580,859. The expression constructs can also comprise at both ends specific oligonucleotide sequences, which may be utilized as primers to amplify the cDNAs by polymerase chain reaction (PCR). The design of the primer sequences for DNA amplification and the ligation of the primer sequences to the cDNAs can be carried out by any methods known in the art, including those described above employing linkers and adaptors. The amplification can be carried out, *e.g.*, by use of a Perkin-Elmer Cetus thermal cycler and Taq polymerase (Gene Amp™). Such a library of cDNA expression constructs can be amplified and maintained *in vitro*, without the use of DNA sequences that propagate the polynucleotide within living cells. Depending on needs, an aliquot of the cDNA expression library can be thawed and introduced directly into host cells. Such expression constructs can be used for expression of cDNAs transiently in recombinant host cells.

5.3.2 cDNA Expression Cloning

Described herein are systems of vectors and host cells that can be used for cloning and expression of a cDNA library. An expression vector is a cloning vector that can be used for maintenance and expression of cDNA library in an appropriate host cell. Any cloning vector known in the art can be used to propagate the cDNA library. A variety of cloning vectors may be used in the present invention which include, but are not limited

to, plasmids, cosmids, phage, phagemids, or modified viruses. Typically, such cloning vectors comprise a functional origin of replication for propagation of the vector in an appropriate host cell, one or more restriction endonuclease sites for insertion of the cDNA library, and one or more selection markers. The cloning vector must be used with a compatible host cell which may be derived from a prokaryotic or an eukaryotic organism including but not limited to bacteria, yeasts, insects, mammals, and humans.

Expression constructs and vectors are introduced into host cells for the purpose of expressing the cDNA library. Host cells broadly encompass cells of unicellular organisms, such as bacteria, fungi, and yeast, and of multicellular organisms, such as insects and animals including but not limited to birds, mammals and humans. Host cells may be obtained from private laboratory deposits, public culture collections such as the American Type Culture Collection, or from commercial suppliers.

cDNA expression cloning in a eukaryotic host is advantageous because egg plasma membrane polypeptide(s) can be post-translationally modified and correctly inserted into the plasma membrane. Such modifications (*e.g.*, glycosylation) and processing (*e.g.*, cleavage) of cDNA-encoded proteins may enhance egg plasma membrane polypeptide activity. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins. A eukaryotic host-vector system is preferred, a mammalian host-vector system is more preferred, and a human host-vector system is the most preferred. Preferred mammalian host cells include but are not limited to those derived from humans, monkeys and rodents, such as chinese hamster ovary (CHO) cells, NIH/3T3, COS, HeLa, Daudi, 293, 293-EBNA, VERO, etc. (see, for example, Kriegler M. in "Gene Transfer and Expression: A Laboratory Manual", New York, Freeman & Co. 1990).

Any of the cloning and expression vectors described herein may be synthesized and assembled from known DNA sequences by well known techniques in the art. The regulatory regions and enhancer elements can be of a variety of origins, both natural and synthetic. Some vectors and host cells may be obtained commercially. Non-limiting examples of useful vectors are pCDM8, λDR2 (see Ausubel *et al.*, eds.,1988, Current Protocols in Molecular Biology, Greene Publishing Associates and Wiley Interscience, New York, Appendix, which is incorporated herein by reference). By way of example, an exemplary expression host-vector system is λDR2 which is a lambda bacteriophage-based cloning vector coupled with a mammalian expression plasmid. Advantages of this system include the utilization of highly efficient lambda *in vitro* packaging systems for initially generating a library in E. coli hosts. Size selection may not be required since the packaging system only accepts inserts in a certain size range. Lambda

vectors generally provide greater ease in amplification and storage. The initial library in E. coli may be amplified to produce supercoiled plasmid DNA which may be used in high efficiency transformation methods for introduction into other expression host organisms. For example, λDR2 uses the lox P mediated site-specific recombination to excise the expression vector pDR2 containing a cDNA insert from lambda clones which can recircularize to generate a plasmid. The plasmid pDR2 contains eukaryotic regulatory regions based on the Epstein-Barr virus and selection markers that allows direct introduction of the cDNA inserts as a library into permissive human host cells at high efficiency.

For expression of cDNAs in mammalian host cells, a variety of regulatory regions can be used, for example, the SV40 early and late promoters, the cytomegalovirus (CMV) immediate early promoter, and the Rous sarcoma virus long terminal repeat (RSV-LTR) promoter. Inducible promoters that may be useful in mammalian cells include but are not limited to those associated with the metallothionein II gene, mouse mammary tumor virus glucocorticoid responsive long terminal repeats (MMTV-LTR), β-interferon gene, and Hsp70 gene (Williams *et al.*, 1989, Cancer Res. 49:2735-42 ; Taylor *et al.*, 1990, Mol. Cell Biol., 10:165-75). The efficiency of cDNA expression in a host cell may be enhanced by the inclusion of appropriate transcription enhancer elements in the expression vector, such as those found in SV40 virus, Hepatitis B virus, cytomegalovirus, immunoglobulin genes, metallothionein, β-actin (see Bittner *et al.*, 1987, Methods in Enzymol. 153:516-544; Gorman, 1990, Curr. Op. in Biotechnol. 1:36-47).

In addition, the expression vector may contain selectable or screenable marker genes for initially isolating, identifying or tracking host cells that contain egg plasma membrane polypeptide cDNA. A number of selection systems may be used for mammalian cells, including but not limited to the Herpes simplex virus thymidine kinase (Wigler *et al.*, 1977, Cell 11:223), hypoxanthine-guanine phosphoribosyltransferase (Szybalski and Szybalski, 1962, Proc. Natl. Acad. Sci. USA 248:2026), and adenine phosphoribosyltransferase (Lowy *et al.*, 1980, Cell 22:817) genes can be employed in tk^-, $hgprt^-$ or $aprt^-$ cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for dihydrofolate reductase (dhfr), which confers resistance to methotrexate (Wigler *et al.*, 1980, Natl. Acad. Sci. USA 77:3567; O'Hare *et al.*, 1981, Proc. Natl. Acad. Sci. USA 78:1527); gpt, which confers resistance to mycophenolic acid (Mulligan and Berg, 1981, Proc. Natl. Acad. Sci. USA 78:2072); neomycin phosphotransferase (neo), which confers resistance to the aminoglycoside G-418 (Colberre-Garapin *et al.*, 1981, J. Mol. Biol. 150:1); and hygromycin phosphotransferase (hyg), which confers resistance to

hygromycin (Santerre *et al.*, 1984, Gene 30:147). Other selectable markers, such as but not limited to histidinol and Zeocin™ can also be used.

A number of viral-based expression systems may also be utilized with mammalian cells to make the cDNA libraries. Vectors using DNA virus backbones have been derived from simian virus 40 (SV40) (Hamer *et al.*, 1979, Cell 17:725), adenovirus (Van Doren *et al.*, 1984, Mol Cell Biol 4:1653), adeno-associated virus (McLaughlin *et al.*, 1988, J. Virol. 62:1963), and bovine papillomas virus (Zinn *et al.*, 1982, Proc Natl Acad Sci 79:4897). In cases where an adenovirus is used as an expression vector, the donor DNA sequence may be ligated to an adenovirus transcription/translation control complex, *e.g.*, the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by *in vitro* or *in vivo* recombination. Insertion in a non-essential region of the viral genome (*e.g.*, region E1 or E3) will result in a recombinant virus that is viable and capable of expressing heterologous products in infected hosts. (See *e.g.*, Logan and Shenk, 1984, Proc. Natl. Acad. Sci. (USA) 81:3655-3659). Alternatively, the vaccinia 7.5K promoter may be used (see, *e.g.*, Mackett *et al.*, 1982, Proc. Natl. Acad. Sci. (USA) 79:7415-7419; Mackett *et al.*, 1984, J. Virol. 49:857-864; Panicali *et al.*, 1982, Proc. Natl. Acad. Sci. 79:4927-4931.) In cases where a human host cell is used, vectors based on the Epstein-Barr virus (EBV) origin (OriP) and EBV nuclear antigen 1 (EBNA-1; a trans-acting replication factor) can be used. Such vectors can be used with a broad range of human host cells, *e.g.*, EBO-pCD (Spickofsky *et al.*, 1990, DNA Prot Eng Tech 2:14-18); pDR2 and λDR2 (available from Clontech Laboratories). The expression vector pDR2 carries the EBV origin which confers stable episomal maintenance to the vector when activated by EBNA-1. Extremely high transfection efficiencies up to 10^{-1} can be obtained when pDR2 is transfected into cell lines which express EBNA-1. Host cells can be rendered proficient for high-efficiency transfections by first transfecting the host cells with an expression construct that produces EBNA-1.

cDNA libraries may also be made with a retrovirus-based expression cloning system. Retroviruses, such as Moloney murine leukemia virus, can be used since most of the viral gene sequence can be removed and replaced with the cDNA library while the missing viral functions can be supplied in trans. In contrast to transfection, retroviruses can efficiently infect and transfer genes to a wide range of cell types including, for example, primary hematopoietic cells. Moreover, the host range for infection by a retroviral vector can be manipulated by the choice of envelope used for vector packaging.

For example, a retroviral vector can comprise a 5' long terminal repeat (LTR), a 3' LTR, a packaging signal, a bacterial origin of replication, and a selectable marker. The cDNA is inserted into a position between the 5' LTR and 3' LTR, such that

transcription from the 5' LTR promoter transcribes the cloned cDNA. The 5' LTR comprises a promoter, including but not limited to an LTR promoter, an R region, a U5 region and a primer binding site, in that order. Nucleotide sequences of these LTR elements are well known in the art. A heterologous promoter as well as multiple drug selection markers may also be included in the expression vector to facilitate selection of infected cells. See, McLauchlin *et al.*, 1990, Prog. Nucleic Acid Res. and Molec. Biol. 38:91-135; Morgenstern *et al.*, 1990, Nucleic Acid Res. 18:3587-3596; Choulika *et al.*, 1996, J Virol 70:1792-1798.

Other useful eukaryotic host-vector system may include yeast and insect systems. In yeast, a number of vectors containing constitutive or inducible promoters may be used with *Saccharomyces cerevisiae* (baker's yeast), *Schizosaccharomyces pombe* (fission yeast), *Pichia pastoris*, and *Hansenula polymorpha* (methylotropic yeasts). For a review see Ausubel *et al.*, eds., 1989, Current Protocols in Molecular Biology, Greene Publishing Associates and Wiley Interscience, New York, Ch. 13; Grant *et al.*, 1987, Expression and Secretion Vectors for Yeast, in Methods in Enzymology, Eds. Wu and Grossman, 1987, Acad. Press, N.Y., Vol. 153, pp. 516-544; Glover, 1986, DNA Cloning, Vol. II, IRL Press, Wash., D.C., Ch. 3; and Bitter, 1987, Heterologous Gene Expression in Yeast, Methods in Enzymology, Eds. Berger and Kimmel, Acad. Press, N.Y., Vol. 152, pp. 673-684; and The Molecular Biology of the Yeast *Saccharomyces*, 1982, Eds. Strathern *et al.*, Cold Spring Harbor Press, Vols. I and II.

In an insect system, Autographa californica nuclear polyhydrosis virus (AcNPV) a baculovirus, can be used as a vector to express cDNA in Spodoptera frugiperda cells. The cDNA sequences may be cloned into non-essential regions (for example the polyhedrin gene) of the virus and placed under control of an AcNPV promoter (for example the polyhedrin promoter). These recombinant viruses are then used to infect host cells in which the inserted DNA is expressed (see, *e.g.*, Smith *et al.*, 1983, J Virol 46:584; Smith, U.S. Patent No. 4,215,051.)

The recombinant host cells may be cultured under standard conditions of temperature, incubation time, optical density, and media composition.

Expression constructs containing cloned cDNA can be introduced into the host cell by a variety of techniques known in the art, including but not limited to, for prokaryotic cells, λ-phage packaging and infection, bacterial transformation (Hanahan, 1985, in DNA Cloning, A Practical Approach, 1:109-136), and for eukaryotic cells, calcium phosphate mediated transfection (Wigler *et al.*, 1977, Cell 11:223-232), liposome-mediated transfection (Schaefer-Ridder *et al.*, 1982, Science 215:166-168), electroporation (Wolff *et*

al., 1987, Proc. Natl. Acad. Sci. 84:3344), and microinjection (Cappechi, 1980, Cell 22:479-488).

In a specific embodiment, and ovarian-derived cell line may be used. It is preferable that the type of host cell is non-adherent to surfaces of cell culture containers, such as plastic, so as to facilitate screening methods and harvesting of the cells.

In one embodiment, an SV40 vector and control sequences are utilized, and the resulting cDNA library is introduced into African green monkey cells (COS cells). The cDNA library can be constructed in a vector containing viral control regions, and introduced in mammalian cells by transfection or infection with viral vectors. Cells are distributed on microtiter dishes for screening. The cDNA library can be transiently expressed in mammalian cells. In a preferred embodiment, the cDNA used in constructing the library is prepared from mRNA isolated from the egg plasma membrane polypeptide positive hybrid cells of the invention. In another embodiment, the library is a "subtracted" cDNA library, wherein gene products common to both egg plasma membrane polypeptide positive hybrid cells and egg plasma membrane polypeptide negative hybrid cells are eliminated from the egg plasma membrane polypeptide positive hybrid cell mRNA or cDNA population by means of subtractive hybridization prior to construction of the cDNA library (see Section 5.4.4). In yet another embodiment, the library is a ovarian cell cDNA library, or a subtracted ovarian cell library (Fargnoli *et al.*, 1990, Anal. Biochem., 187:364-73; Wang and Brown, 1991, Proc. Natl. Acad. Sci. 88:11505-09; Lisitsyn, 1995, Trends Genet. 11:303-7; Zeng *et al.*, 1994, Nuc. Acid Res. 22:4381-85; Hakvoort *et al.*, 1996, Nucl. Acids Res. 24:3478-80; Lavery *et al.*, 1997, Proc. Natl. Acad. Sci. USA 13:6831-36).

The cDNA library can be screened directly or indirectly. A number of indirect methods are possible that rely on expression of cDNA insert and screening for its activity by binding assays, immunological methods, or an altered cellular phenotype. egg plasma membrane polypeptide can be isolated indirectly by screening the cDNA expression library for egg plasma membrane polypeptide activity, such as ligand binding or egg plasma membrane polypeptide antibody-binding activity. For example, egg plasma membrane polypeptide antibodies can be labeled with a detectable compound, such as a radioactive, fluorescent or biotinylated compound, and used as probes to screen bacterial colonies that have been induced to express cDNA inserts.

In one embodiment, the eukaryotic expression library can be screened by panning (Seed, 1987, Proc. Natl. Acad. Sci. USA 84:3365-69). Using this technique, culture dishes are pre-coated with antibody, which can bind to cells that express egg plasma membrane polypeptide. Alternatively, culture dishes may be coated with egg plasma membrane polypeptide protein, which also can bind to cells that express egg plasma

membrane polypeptide. Non-adherent cells can be rinsed away, and selected cells can be isolated and their inserts can be further analyzed. Alternatively, a cDNA library can be screened directly by hybridization. An oligonucleotide probe designed from the sequence of the egg plasma membrane polypeptide protein. Egg plasma membrane polypeptide(s) can be purified and sequenced, as described in Section 5.2, *supra*. Protein sequence information is then used to design degenerate oligonucleotides containing all possible codons for egg plasma membrane polypeptide amino acids. Sequence information from various regions of the protein can be used to generate a series of such degenerate pools of oligonucleotides. Thus, each oligonucleotide pool contains some sequences that are complementary to egg plasma membrane polypeptide gene sequences. Such degenerate oligonucleotide pools can be used to screen a cDNA library, prepared as described herein, *supra*.

In another embodiment, the expression library can be screened with fluorescently or magnetically labeled egg plasma membrane polypeptide antibody, using cell sorting methods known in the art. Such labeling and sorting methods are described in detail in Section 5.2, *supra*.

For cDNA expression in prokaryotic cells, cDNA can be cloned into a plasmid or phage vector. Vectors based on *E. coli* are the most popular and versatile systems for high level expression of foreign proteins (Makrides, 1996, Microbiol. Rev., 60:512-538). Non-limiting examples of regulatory regions that can be used for expression in E. coli may include but not limited to lac, trp, lpp, phoA, recA, tac, T3, T7 and λP_L (Makrides, 1996, Microbiol Rev, 60:512-538). Non-limiting examples of prokaryotic expression vectors may include the λgt vector series such as λgt11 (Huynh *et al.*, 1984 in "DNA Cloning Techniques", Vol. I: A Practical Approach (D. Glover, ed.), pp. 49-78, IRL Press, Oxford), and the pET vector series (Studier *et al.*, 1990, Methods Enzymol., 185:60-89). λgt11 is particularly advantageous for this purpose. The phage contains the temperature sensitive repressor λc1857 which is inactive at 42°C and the expression of the cDNA insert is under the control of the lac operon (Young and Davis, 1983, Science 222:778-782). Proteins may be induced by shifting temperature to 42°C. In this way, the expression of foreign proteins which are potentially deleterious or lethal to cell growth can be tightly controlled while bacterial colonies are growing at 37°C. Furthermore, in this system, cloning of cDNA insert interrupts the β-galactosidase gene, so that recombinants can be readily identified by addition of the gratuitous *lac* operon inducer isopropyl thio-β-D-galactopyranoside (IPTG) and assaying for β-galactosidase activity, by methods well known in the art, such as plating on X-gal.

Expression constructs containing cloned cDNA can be introduced into the prokaryotic host cell by a variety of techniques known in the art, including but not limited to, λ-phage packaging and infection, transduction and transformation (Hanahan, 1985, in DNA Cloning, A Practical Approach, 1:109-136). Bacteria is infected with phage or transformed with plasmid carrying the cDNA library, plated on LB agar plates, and induced to express cDNA inserts.

However, a potential drawback of a prokaryotic host-vector system is the inability to perform many of the post-translational processing, folding and insertion into membranes normally required of cell surface receptors.

A specific cDNA insert can be detected and isolated by inducing expression of the cDNA inserts and utilizing screening methods that rely on detection of protein activity. Such methods include filter binding to a labeled ligand or immunological methods to detect antibody binding.

For example, in a preferred embodiment, egg surface polypeptide can be isolated by screening the cDNA expression library for egg surface polypeptide activity, such as egg surface polypeptide antibody-binding or ligand binding. For example, egg surface polypeptide antibodies can be labeled with a detectable compound, such as a radioactive, fluorescent or biotinylated compound can be used as probes to screen induced proteins colonies attached to filters. Egg surface polypeptide antibody mixture is incubated using conditions that promote binding and developed as described above to detect egg surface polypeptide clones. Alternatively, immunological methods are used to detect antibody.

In another embodiment, a cDNA library can be screened for egg surface polypeptide expression in frog oocytes. Frog oocytes are advantageous for this purpose because their large size and (1-1.2 mm) and their abundance of protein translation machinery. In addition, insertion of receptor proteins can be inserted into membranes readily screened for activity. A cDNA library is constructed in a vector containing T3, T7, SP6 or other RNA polymerase promoter located on either side of a polylinker containing cloning sites for insertion of cDNA. cDNAs can be prepared and inserted into the vector, the library is amplified, and plasmid DNA is isolated and linearized by cutting with a restriction endonuclease whose site is in the polylinker. Run-off in transcriptions are performed *in vitro*, by addition of nucleotides and the appropriate polymerase, and mRNAs are injected into oocytes. After allowing for translation, oocytes are incubated with ligand labeled with radioactive, fluorescent, or otherwise detectable compound. Sublibraries displaying a positive signal are further divided, plasmid DNA is isolated, *in vitro* transcribed and injected until a single clone is isolated.

Any of the above described methods can be used to identify egg surface polypeptide gene candidates. Positive clones can be isolated, purified and the sequence of their inserts can be determined. Such purified inserts can be used for the isolation of full length and genomic sequences, and for the expression of egg surface polypeptide proteins as described below.

5.4 EXPRESSION OF EGG SURFACE POLYPEPTIDE NUCLEIC ACIDS

The nucleotide sequence coding for an egg surface polypeptide protein or a functionally active analog or fragment or other derivative thereof, can be inserted into an appropriate expression vector, *i.e.*, a vector which contains the necessary elements for the transcription and translation of the inserted protein-coding sequence. The necessary transcriptional and translational signals can also be supplied by the native egg surface polypeptide gene and/or its flanking regions. A variety of host-vector systems can be utilized to express the protein-coding sequence. These include but are not limited to mammalian cell systems infected with virus (*e.g.*, vaccinia virus, adenovirus, etc.); insect cell systems infected with virus (*e.g.*, baculovirus); microorganisms such as yeast containing yeast vectors, or bacteria transformed with bacteriophage, DNA, plasmid DNA, or cosmid DNA. The expression elements of vectors vary in their strengths and specificities. Depending on the host-vector system utilized, any one of a number of suitable transcription and translation elements can be used. In yet another embodiment, a fragment of an egg surface polypeptide protein comprising one or more domains of the egg surface polypeptide protein is expressed.

Any of the methods previously described for the insertion of DNA fragments into a vector can be used to construct expression vectors containing a chimeric gene comprising of appropriate transcriptional/translational control signals and the protein coding sequences. These methods can include *in vitro* recombinant DNA and synthetic techniques and *in vivo* recombinants (genetic recombination). Expression of a nucleic acid sequence encoding an egg surface polypeptide protein or peptide fragment can be regulated by a second nucleic acid sequence so that the egg surface polypeptide protein or peptide is expressed in a host transformed with the recombinant DNA molecule. For example, expression of an egg surface polypeptide protein can be controlled by any promoter/enhancer element known in the art. Promoters which can be used to control egg surface polypeptide gene expression include, but are not limited to, the SV40 early promoter region (Benoist and Chambon, 1981, Nature 290:304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto et al., 1980, Cell 22:787-797), the herpes thymidine kinase promoter (Wagner et al., 1981, Proc. Natl. Acad.

Sci. U.S.A. 78:1441-1445), the regulatory sequences of the metallothionein gene (Brinster et al., 1982, Nature 296:39-42); prokaryotic promoters such as the β-lactamase promoter (Villa-Kamaroff et al., 1978, Proc. Natl. Acad. Sci. U.S.A. 75:3727-3731), or the lac promoter (DeBoer et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:21-25); see also "Strategies for Achieving High Level Expression of Genes in *Escherichia coli*" in Microbiological Reviews, 1996, 60:514; plant expression vectors comprising the nopaline synthetase promoter region (Herrera-Estrella et al., Nature 303:209-213) or the cauliflower mosaic virus 35S RNA promoter (Gardner et al., 1981, Nucl. Acids Res. 9:2871), and the promoter of the photosynthetic enzyme ribulose biphosphate carboxylase (Herrera-Estrella et al., 1984, Nature 310:115-120); promoter elements from yeast or other fungi such as the Gal 4 promoter, the ADC (alcohol dehydrogenase) promoter, PGK (phosphoglycerol kinase) promoter, alkaline phosphatase promoter, and the following animal transcriptional control regions, which exhibit tissue specificity and have been utilized in transgenic animals: elastase I gene control region which is active in pancreatic acinar cells (Swift et al., 1984, Cell 38:639-646; Ornitz et al., 1986, Cold Spring Harbor Symp. Quant. Biol. 50:399-409; MacDonald, 1987, Hepatology 7:425-515); a gene control region which is active in pancreatic beta cells (Hanahan, 1985, Nature 315:115-122), an immunoglobulin gene control region which is active in lymphoid cells (Grosschedl et al., 1984, Cell 38:647-658; Adames et al., 1985, Nature 318:533-538; Alexander et al., 1987, Mol. Cell. Biol. 7:1436-1444), mouse mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder et al., 1986, Cell 45:485-495), albumin gene control region which is active in liver (Pinkert et al., 1987, Genes and Devel. 1:268-276), alpha-fetoprotein gene control region which is active in liver (Krumlauf et al., 1985, Mol. Cell. Biol. 5:1639-1648; Hammer et al., 1987, Science 235:53-58; α1-antitrypsin gene control region which is active in the liver (Kelsey et al., 1987, Genes and Devel. 1:161-171), β-globin gene control region which is active in myeloid cells (Mogram et al., 1985, Nature 315:338-340; Kollias et al., 1986, Cell 46:89-94; myelin basic protein gene control region which is active in oligodendrocyte cells in the brain (Readhead et al., 1987, Cell 48:703-712); myosin light chain-2 gene control region which is active in skeletal muscle (Sani, 1985, Nature 314:283-286), and gonadotropic releasing hormone gene control region which is active in the hypothalamus (Mason et al., 1986, Science 234:1372-1378).

Many of the vectors and prokaryotic/eukaryotic host cell systems described supra for constructing gene expression libraries can be used for egg surface polypeptide expression.

In a specific embodiment, a vector is used that comprises a promoter operably linked to an egg surface polypeptide gene nucleic acid, one or more origins of

replication, and, optionally, one or more selectable markers (*e.g.*, an antibiotic resistance gene).

In a specific embodiment, an expression construct is made by subcloning an egg surface polypeptide coding sequence into the EcoRI restriction site of each of the three pGEX vectors (glutathione S-transferase expression vectors; Smith and Johnson, 1988, Gene 7:31-40). This allows for the expression of the egg surface polypeptide protein product from the subclone in the correct reading frame.

Expression vectors containing egg surface polypeptide gene inserts can be identified by three general approaches: (a) nucleic acid hybridization; (b) presence or absence of "marker" gene functions; and (c) expression of inserted sequences. In the first approach, the presence of an egg surface polypeptide gene inserted in an expression vector can be detected by nucleic acid hybridization using probes comprising sequences that are homologous to an inserted egg surface polypeptide gene. In the second approach, the recombinant vector/host system can be identified and selected based upon the presence or absence of certain "marker" gene functions (*e.g.*, thymidine kinase activity, resistance to antibiotics, transformation phenotype, occlusion body formation in baculovirus, etc.) caused by the insertion of an egg surface polypeptide gene in the vector. For example, if the egg surface polypeptide gene is inserted within the marker gene sequence of the vector, recombinants containing the egg surface polypeptide insert can be identified by the absence of the marker gene function. In the third approach, recombinant expression vectors can be identified by assaying the egg surface polypeptide product expressed by the recombinant. Such assays can be based, for example, on the physical or functional properties of the egg surface polypeptide protein *in vitro* assay systems, *e.g.*, binding with anti-egg surface polypeptide protein antibody.

Once a particular recombinant DNA molecule is identified and isolated, several methods known in the art can be used to propagate it. Once a suitable host system and growth conditions are established, recombinant expression vectors can be propagated and prepared in quantity. As previously explained, the expression vectors which can be used include, but are not limited to, the following vectors or their derivatives: human or animal viruses such as vaccinia virus or adenovirus; insect viruses such as baculovirus; yeast vectors; bacteriophage vectors (*e.g.*, lambda phage), and plasmid and cosmid DNA vectors, to name but a few.

In addition, a host cell strain can be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Expression from certain promoters can be elevated in the presence of certain inducers; thus, expression of the genetically engineered egg surface polypeptide protein can

be controlled. Furthermore, different host cells have characteristic and specific mechanisms for the translational and post-translational processing and modification (*e.g.*, glycosylation, phosphorylation of proteins. Appropriate cell lines or host systems can be chosen to ensure the desired modification and processing of the foreign protein expressed. For example, expression in a bacterial system can be used to produce a non-glycosylated core protein product. Expression in yeast will produce a glycosylated product. Expression in mammalian cells can be used to ensure "native" glycosylation of a heterologous protein. Furthermore, different vector/host expression systems can effect processing reactions to different extents.

In other specific embodiments, the egg surface polypeptide, fragment, analog, or derivative can be expressed as a fusion, or chimeric protein product (comprising the protein, fragment, analog, or derivative joined via a peptide bond to a heterologous protein sequence of a different protein). Such a chimeric product can be made by ligating the appropriate nucleic acid sequences encoding the desired amino acid sequences to each other by methods known in the art, in the proper coding frame, and expressing the chimeric product by methods commonly known in the art. Alternatively, such a chimeric product can be made by protein synthetic techniques, *e.g.*, by use of a peptide synthesizer (particularly if some protein sequence is available). The recombinant egg surface polypeptide may not be fully functional for a number of reasons. For example, egg surface polypeptide may be modified *in vivo*. A functional group(s), such as methylation, phosphorylation, or glycosylation, may be added posttranslationally, and play important role(s) in receptor function. Further, egg surface polypeptide may be composed of more than a single polypeptide subunit. In this case recombinant egg surface polypeptide will lack the full activity of the native protein.

5.5 GENERATION OF ANTIBODIES TO EGG SURFACE POLYPEPTIDES

According to the invention, an egg surface protein, its fragments or other derivatives, or analogs thereof, may be used as an immunogen to generate antibodies which immunospecifically bind such an immunogen. Such antibodies include but are not limited to polyclonal, monoclonal, chimeric, single chain, Fab fragments, and an Fab expression library. In a specific embodiment, antibodies to a human egg surface protein are produced. In another embodiment, antibodies to a domain (*e.g.*, the extracelluar domain released by treatment with PI-PLC) of an egg surface protein are produced. In a specific embodiment, fragments of an egg surface protein identified as hydrophilic are used as immunogens for antibody production.

Various procedures known in the art may be used for the production of polyclonal antibodies to an egg surface protein or derivative or analog thereof. In a particular embodiment, rabbit polyclonal antibodies to an epitope of an egg surface protein or fragment, can be obtained. For the production of antibody, various host animals, including but not limited to rabbits, mice, rats, etc can be immunized by injection with the native egg surface proteins, or a synthetic version, or derivative (*e.g.*, fragment) thereof. Various adjuvants may be used to increase the immunological response, depending on the host species, and including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and corynebacterium parvum.

For preparation of monoclonal antibodies directed toward an egg surface protein sequence or analog thereof, any technique which provides for the production of antibody molecules by continuous cell lines in culture may be used. For example, the hybridoma technique originally developed by Kohler and Milstein (1975, Nature 256:495-497), as well as the trioma technique, the human B-cell hybridoma technique (Kozbor et al., 1983, Immunology Today 4:72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., 1985, in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). In an additional embodiment of the invention, monoclonal antibodies can be produced in germ-free animals utilizing recent technology (PCT/US90/02545). According to the invention, human antibodies may be used and can be obtained by using human hybridomas (Cote et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:2026-2030) or by transforming human B cells with EBV virus *in vitro* (Cole et al., 1985, in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, pp. 77-96). In fact, according to the invention, techniques developed for the production of "chimeric antibodies" (Morrison et al., 1984, Proc. Natl. Acad. Sci. U.S.A. 81:6851-6855; Neuberger et al., 1984, Nature 312:604-608; Takeda et al., 1985, Nature 314:452-454) by splicing the genes from a mouse antibody molecule specific for egg surface proteins together with genes from a human antibody molecule of appropriate biological activity can be used; such antibodies are within the scope of this invention.

According to the invention, techniques described for the production of single chain antibodies (U.S. Patent 4,946,778) can be adapted to produce egg surface protein-specific single chain antibodies. An additional embodiment of the invention utilizes the techniques described for the construction of Fab expression libraries (Huse et al., 1989,

Science 246:1275-1281) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity for egg surface proteins, derivatives, or analogs.

Antibody fragments which contain the idiotype of the molecule can be generated by known techniques. For example, such fragments include but are not limited to: the $F(ab')_2$ fragment which can be produced by pepsin digestion of the antibody molecule; the Fab' fragments which can be generated by reducing the disulfide bridges of the $F(ab')_2$ fragment, the Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent, and Fv fragments.

In the production of antibodies, screening for the desired antibody can be accomplished by techniques known in the art, *e.g.* ELISA (enzyme-linked immunosorbent assay). For example, to select antibodies which recognize a specific domain of an egg surface protein, one may assay generated hybridomas for a product which binds to an egg surface protein fragment containing such domain. For selection of an antibody that specifically binds a first egg surface protein homolog but which does not specifically bind a different egg surface protein homolog, one can select on the basis of positive binding to the first egg surface protein homolog and a lack of binding to the second egg surface protein homolog.

Antibodies specific to a domain of an egg surface proteins are also provided.

The foregoing antibodies can be used in methods known in the art relating to the localization and activity of the egg surface proteins of the invention, *e.g.*, for imaging these proteins, measuring levels thereof in appropriate physiological samples, in diagnostic methods, etc.

In another embodiment of the invention (see *infra*), anti-egg surface antibodies and fragments thereof containing the extracellular domain are useful contraceptive vaccines.

5.6 ASSAYS TO IDENTIFY COMPOUNDS THAT MODULATE EGG SURFACE POLYPEPTIDE ACTIVITY

The egg surface proteins of the invention are involved in mediating sperm-egg fusion via a direct interaction between egg surface proteins and a sperm ligand. Thus, the present invention relates to *in vitro* and *in vivo* assay systems, described in the subsections below, which can be used to identify compounds or compositions that modulate the activity of egg surface polypeptide and its interaction with a sperm ligand. Such molecules, such as peptides or non-protein molecules, including organic or inorganic small molecules, large molecules, antibodies, and nucleotide sequences may bind egg surface polypeptide with differing affinities. Such molecules can serve as powerful modulators of fertilization *in vivo*, and can be used therapeutically to modulate the fertility. The screening

assays of the present invention may also be used to identify compounds or compositions that modulate the interaction of egg surface polypeptides with its binding partners, as identified herein.

Methods to screen potential agents for their ability to disrupt or moderate egg surface polypeptide expression and activity can be designed based on the Inventor's discovery of egg surface polypeptides, such as M70, M35/45, H25/40, and GP1-linked ZP3, and their role in egg-sperm fusion and fertilization. The egg surface polypeptide proteins, nucleic acids, and derivatives can be used in screening assays to detect molecules that specifically bind to egg surface polypeptide proteins, derivatives, or nucleic acids, and thus have potential use as agonists or antagonists of egg surface polypeptide, to modulate fertility. In a preferred embodiment, such assays are performed to screen for molecules with potential utility in modulating fertility, thereby useful as contraceptive or sterilization agents. For example, recombinant cells expressing egg surface polypeptide nucleic acids can be used to recombinantly produce an egg surface polypeptide in these assays, to screen for molecules that bind to the egg surface polypeptide. Similar methods can be used to screen for molecules that bind to egg surface polypeptide derivatives or nucleic acids. Methods that can be used to carry out the foregoing are commonly known in the art.

In principle, many methods known to those of skill in the art, can be readily adapted in designing the assays of the present invention. Screening methodologies are well known in the art (see *e.g.*, PCT International Publication No. WO 96/34099, published October 31, 1996, which is incorporated by reference herein in its entirety).

The screening assays, described herein, can be used to identify compounds and compositions including peptides and organic, non-protein molecules that modulate egg surface polypeptide activity. Recombinant, synthetic, and otherwise exogenous compounds may have binding capacity and, therefore, may be candidates for pharmaceutical agents. Alternatively, the proteins and compounds include endogenous cellular components which interact with the identified genes and proteins in vivo. Such endogenous components may provide new targets for pharmaceutical and therapeutic interventions.

Alternatively, any of a variety of exogenous compounds, both naturally occurring and/or synthetic (*e.g.*, libraries of small molecules or peptides), may be screened for binding capacity.

Thus, in a preferred embodiment, both naturally occurring and/or synthetic compounds (*e.g.*, libraries of small molecules or peptides), may be screened for modulating egg surface polypeptide activity. In another series of embodiments, cell lysates or tissue homogenates may be screened for proteins or other compounds which bind to one of the normal or mutant egg surface polypeptide genes and the egg surface polypeptide proteins.

Within the broad category of *in vitro* selection methods, several types of method are likely to be particularly convenient and/or useful for screening test agents. These include, but are not limited to, methods which measure binding of a compound to a egg surface polypeptide, methods which measure a change in the ability of egg surface polypeptide or egg surface polypeptide-positive hybrid cells to interact with an egg surface polypeptide antibody or ligand *in vitro*, and methods which measure the activity or expression of "reporter" protein, that is, an enzyme or other detectable or selectable protein, which has been placed under the control of an egg surface polypeptide gene control region.

The assays of the present invention may be first optimized on a small scale (i.e., in test tubes), and then scaled up for high-throughput assays. The screening assays of the present may be performed *in vitro*, i.e. in test tubes, using purified components or cell lysates. The screening assays of the present invention may also be carried out in intact cells in culture and in animal models. In accordance with the present invention, test compounds which are shown to modulate the activity of egg surface polypeptide *in vitro*, as described herein, will further be assayed *in vivo* in cultured cells and animal models to determine if the test compound has the similar effects *in vivo* and to determine the effects of the test compound on T-cell cytotoxicity, antigen presentation, tumor progression, the accumulation or degradation of positive and negative regulators, cellular proliferation, etc.

In accordance with this embodiment, the systems described below may be formulated into kits. To this end, cells expressing egg surface polypeptide or cell lysates thereof can be packaged in a variety of containers, *e.g.*, vials, tubes, microtiter well plates, bottles, and the like. Other reagents can be included in separate containers and provided with the kit; *e.g.*, positive control samples, negative control samples, buffers, cell culture media, etc.

In one embodiment of the present invention, peptide libraries may be used to screen for agonists or antagonists of egg surface polypeptide. Diversity libraries, such as random or combinatorial peptide or non-peptide libraries can be screened for molecules that specifically bind to egg surface polypeptide. Many libraries are known in the art that can be used, *e.g.*, chemically synthesized libraries, recombinant (*e.g.*, phage display libraries), and *in vitro* translation-based libraries.

Examples of chemically synthesized libraries are described in Fodor et al., 1991, Science 251:767-773; Houghten et al., 1991, Nature 354:84-86; Lam et al., 1991, Nature 354:82-84; Medynski, 1994, Bio/Technology 12:709-710; Gallop et al., 1994, J. Medicinal Chemistry 37(9):1233-1251; Ohlmeyer et al., 1993, Proc. Natl. Acad. Sci. USA 90:10922-10926; Erb et al., 1994, Proc. Natl. Acad. Sci. USA 91:11422-11426; Houghten et al., 1992, Biotechniques 13:412; Jayawickreme et al., 1994, Proc. Natl. Acad. Sci. USA

91:1614-1618; Salmon et al., 1993, Proc. Natl. Acad. Sci. USA 90:11708-11712; PCT Publication No. WO 93/20242; and Brenner and Lerner, 1992, Proc. Natl. Acad. Sci. USA 89:5381-5383.

Examples of phage display libraries are described in Scott and Smith, 1990, Science 249:386-390; Devlin et al., 1990, Science, 249:404-406; Christian, et al., 1992, J. Mol. Biol. 227:711-718; Lenstra, 1992, J. Immunol. Meth. 152:149-157; Kay et al., 1993, Gene 128:59-65; and PCT Publication No. WO 94/18318 dated August 18, 1994.

By way of examples of non-peptide libraries, a benzodiazepine library (*see e.g.*, Bunin et al., 1994, Proc. Natl. Acad. Sci. USA 91:4708-4712) can be adapted for use. Peptoid libraries (Simon et al., 1992, Proc. Natl. Acad. Sci. USA 89:9367-9371) can also be used. Another example of a library that can be used, in which the amide functionalities in peptides have been permethylated to generate a chemically transformed combinatorial library, is described by Ostresh et al. (1994, Proc. Natl. Acad. Sci. USA 91:11138-11142).

Screening the libraries can be accomplished by any of a variety of commonly known methods. See, *e.g.*, the following references, which disclose screening of peptide libraries: Parmley and Smith, 1989, Adv. Exp. Med. Biol. 251:215-218; Scott and Smith, 1990, Science 249:386-390; Fowlkes et al., 1992; BioTechniques 13:422-427; Oldenburg et al., 1992, Proc. Natl. Acad. Sci. USA 89:5393-5397; Yu et al., 1994, Cell 76:933-945; Staudt et al., 1988, Science 241:577-580; Bock et al., 1992, Nature 355:564-566; Tuerk et al., 1992, Proc. Natl. Acad. Sci. USA 89:6988-6992; Ellington et al., 1992, Nature 355:850-852; U.S. Patent No. 5,096,815, U.S. Patent No. 5,223,409, and U.S. Patent No. 5,198,346, all to Ladner et al.; Rebar and Pabo, 1993, Science 263:671-673; and PCT Publication No. WO 94/18318.

In a preferred embodiment, screening can be carried out by contacting the library members with egg surface polypeptide protein (or nucleic acid or derivative) immobilized on a solid phase surface and harvesting those library members that bind to the protein (or nucleic acid or derivative). In a specific embodiment, a library can be screened by passing phage from a continuous phage display library through a column containing purified egg surface polypeptide linked to a solid phase surface, such as plastic beads. By altering the stringency of the washing buffer, it is possible to enrich for phage that express peptides with high affinity for egg surface polypeptide. Phage isolated from the column can be cloned and the affinities of the short peptides can be measured directly. Sequences for more than one oligonucleotide can be combined to test for even higher affinity binding to egg surface polypeptide. Knowing which amino acid sequences confer the strongest binding to egg surface polypeptide, computer models can be used to identify the molecular contacts between egg surface polypeptide and ligand. This will allow the design of non-

protein compounds which mimic those contacts. Such a compound may have the same activity of the peptide and can be used therapeutically, having the advantage of being efficient and less costly to produce.

In another specific embodiment of this aspect of the invention, the solid support is egg surface polypeptide protein (or nucleic acid or derivative) immobilized on a microtiter dish. Cells that express library members are cultivated under conditions that allow expression of the library members in the microtiter dish. Library members that bind to the protein (or nucleic acid or derivative) are harvested. Such methods, are described by way of example in Parmley and Smith, 1988, Gene 73:305-318; Fowlkes et al., 1992, BioTechniques 13:422-427; PCT Publication No. WO 94/18318; and in references cited hereinabove.

In another embodiment of the present invention, interactions between egg surface polypeptide and a test compound may be assayed *in vitro*. Known or unknown molecules are assayed for specific binding to egg surface polypeptide nucleic acids, proteins, or derivatives under conditions conducive to binding, and then molecules that specifically bind to egg surface polypeptide are identified. The two components can be measured in a variety of ways. One approach is to label one of the components with an easily detectable label, place it together with a test component(s) under conditions that allow binding to occur, perform a separation step which separates bound labeled component from unbound labeled component, and then measure the amount of bound component. In one embodiment, egg surface polypeptide can be labeled and added to a test agent, using conditions that allow binding to occur. Binding of the test agent can be determined using polyacrylamide gel analysis to compare complexes formed in the presence and absence of the test agent.

In another embodiment of the present invention, the screening may be performed by adding the labeled egg surface polypeptide to *in vitro* translation systems such as a rabbit reticulocyte lysate (RRL) system and then proceeding with the binding reaction. *In vitro* translation-based libraries include but are not limited to those described in PCT Publication No. WO 91/05058 dated April 18, 1991; and Mattheakis et al., 1994, Proc. Natl. Acad. Sci. USA 91:9022-9026.

In another embodiment, binding of egg surface polypeptide to a test agent may be assayed in intact cells in culture or in animal models in the presence and absence of the test compound. For example, a labeled test agent may be mixed with eggs in culture, or to crude extracts obtained from animal tissue samples, and the test compound may be added. Binding can be assayed using microscopy or confocal microscopy, for example. In yet another embodiment, the test agent may be assayed in intact cells in animal models. A

labeled test agent may be administered directly to an animal. The uptake of the test agent may be measured. For these assays, host cells to which the test compound is added may be genetically engineered to express egg surface polypeptide and its target interactor (such as an egg surface polypeptide antibody or an egg surface polypeptide ligand) which may be transient, induced or constitutive, or stable. For the purposes of the screening methods of the present invention, a wide variety of host cells may be used including, but not limited to, tissue culture cells, mammalian cells, yeast cells, and bacteria. Each cell type has its own set of advantages and drawbacks. Mammalian cells, such as egg cells which express the egg surface polypeptides of the invention, may be a preferred cell type in which to carry out the assays of the present invention. Bacteria and yeast are relatively easy to cultivate but process proteins differently from mammalian cells.

In a specific embodiment, the invention provides for a method for detecting the interaction between egg surface polypeptide and a known potential ligand, such as a sperm integrin. Insect cells can be infected with baculoviruses co-expressing egg surface polypeptide and the known sperm integrin, and cell extracts can be prepared and analyzed for protein-protein interactions. Protein-protein interactions can be analyzed by methods known in the art, such as Western blotting or immune precipitation using egg surface polypeptide specific antibodies together with an anti-integrin antibody, and analyzing complexes by polyacrylamide gel electrophoresis.

The invention further provides methods for screening cells having egg surface polypeptide proteins (or fragments thereof) as one of their cell surface membrane components for known cell surface molecules as potential ligands. For example, cells engineered to express egg surface polypeptide nucleic acids can be used to recombinantly produce egg surface polypeptide proteins either wild-type or dominant negative mutants in cells that also express a putative egg surface polypeptide binding partner molecule. Potential candidates for an egg surface polypeptide binding partner include, but are not limited to, such known substrates as sperm integrin. Extracts can also be used to test whether the presence of egg surface polypeptide increases or decreases the level of the potential binding partner.

In another embodiment, the two-hybrid system for selecting interacting proteins or peptides in yeast (Fields and Song, 1989, Nature 340:245-246; Chien et al., 1991, Proc. Natl. Acad. Sci. USA 88:9578-9582) can be used to identify molecules that specifically bind to egg surface polypeptide protein or derivative.

In yet another embodiment of the present invention, peptide libraries may be used to identify for unknown potential ligands of egg surface polypeptide. Diversity libraries, such as random or combinatorial peptide libraries can be screened for molecules

that specifically bind to egg surface polypeptide. Many libraries are known in the art that can be used, *e.g.*, chemically synthesized libraries, recombinant (*e.g.*, phage display libraries), and *in vitro* translation-based libraries.

Once a substrate or interacting protein is identified, then one can assay for modulators of the egg surface polypeptide interaction with such a protein. The present invention provides for methods of detecting agonists and antagonists of such interactions.

Recombinant egg surface polypeptide and putative agonist or antagonist molecules can be incubated together, under conditions that allow binding to occur, such as 37°C for 30 minutes. Protein-protein complex formation can be detected by acrylamide gel analysis, by methods known in the art. This assay can be used to identify modulators of interactions of sperm and egg surface proteins involved in fertilization.

Purified or partially purified components which have been determined to interact with one another by the methods described above can be placed under conditions in which the interaction between them would normally occur, with and without the addition of the test agent, and the procedures previously established to analyze the interaction can be used to assess the impact of the test agent. In this approach, the purified or partially purified components may be prepared by fractionation of extracts of eggs, or they may be obtained by expression of cloned genes or cDNAs or fragments thereof, optionally followed by purification of the expressed material.

5.7 VACCINES AGAINST EGG SURFACE PROTEINS

Mouse or hamster egg surface proteins can be used in any of a variety of vaccines in human and non-human animals. The present invention encompasses vaccines useful for contraception. In one aspect of the invention, egg surface polypeptides involved in sperm-egg fusion, such as M70, M35/45, H25/40, and GP1-linked ZP3, are delivered to a subject to elicit an active immune response. The vaccine acts as a temporary and reversible antagonist of the function of the egg surface proteins of the invention. For example, such vaccines could be used for active immunization of a subject, to raise an antibody response to temporarily block the sperm's access to the egg plasma antigen. In one aspect of the invention, an antigen could be administered at a certain period of the month, for example during ovulation of a female subject to block fertilization.

In another aspect of the invention, egg surface polypeptides involved in sperm-egg fusion, such as GPI-linked ZP3, M70, M35/45, H25/40, and GP1-linked ZP3, are useful as vaccines for permanent sterilization of a subject. Such vaccines can be used to elicit a T-cell mediated attack on the eggs, having an othoritic effect, useful as a method for

irreversible sterilization. Methods for generating T-cell specific responses, such as adoptive immunotherapy, are well known in the art (see, for example, Vaccine Design, Michael F. Powell and Mark J. Newman Eds., Plenum Press, New York, 1995, pp 847-867). Such techniques may be particular useful for vetinary contraceptive or sterilization purposes, where a single dose vaccination may be desirable.

5.7.1 Vaccine Formulations and Methods of Administration

Egg surface protein antigens can be produced in large amounts and purified for use in vaccine preparations. The egg surface proteins of the invention also have utility in immunoassays, e.g., to detect or measure in a sample of body fluid from a vaccinated subject the presence of antibodies to the antigen, and thus to diagnose and/or to monitor immune response of the subject subsequent to vaccination.

The preparation of vaccines containing an immunogenic polypeptide as the active ingredient is known to one skilled in the art (see, for example, Vaccine Design, Michael F. Powell and Mark J. Newman Eds., Plenum Press, New York, 1995, pp 821-902)

5.7.1.1 Determination of Vaccine Efficacy

The immunopotency of egg surface protein antigens can be determined by monitoring the immune response in test animals following immunization with the egg surface protein antigen, or by use of any immunoassay known in the art. Generation of a humoral (antibody) response and/or cell-mediated immunity, may be taken as an indication of an immune response. Test animals may include mice, hamsters, dogs, cats, monkeys, rabbits, chimpanzees, etc., and eventually human subjects.

Methods of introducing the vaccine may include oral, intravaginal, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, and via scarification (scratching through the top layers of skin, *e.g.*, using a bifurcated needle) or any other standard routes of immunization. The immune response of the test subjects can be analyzed by various approaches such as: the reactivity of the resultant immune serum to the egg protein antigen, as assayed by known techniques, e.g., immunosorbent assay (ELISA), immunoblots, radioimmunoprecipitations, etc., or in the case where the egg protein antigen displays antigenicity or immunogenicity, by protection of the immunized host against fertilization in the immunized host.

As one example of suitable animal testing of an egg surface protein vaccine, the vaccine of the invention may be tested in rabbits for the ability to induce an antibody response to the egg surface protein antigen. Male specific-pathogen-free (SPF) young adult New Zealand White rabbits may be used. The test group each receives a fixed

concentration of the vaccine. A control group receives an injection of 1 mM Tris-HCl pH 9.0 without the egg surface protein antigen.

Blood samples may be drawn from the rabbits every one or two weeks, and serum analyzed for antibodies to the egg surface protein. The presence of antibodies specific for the antigen may be assayed, e.g., using an ELISA.

5.7.1.2 Vaccine Formulations

Suitable preparations of such vaccines include injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, suspension in, liquid prior to injection, may also be prepared. The preparation may also be emulsified, or the polypeptides encapsulated in liposomes. The active immunogenic ingredients are often mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water saline, dextrose, glycerol, ethanol, or the like and combinations thereof. In addition, if desired, the vaccine preparation may also include minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, and/or adjuvants which enhance the effectiveness of the vaccine.

Examples of adjuvants which may be effective, include, but are not limited to: aluminum hydroxide, N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acetyl-nor-muramyl-L-alanyl-D-isoglutamine, N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-2'-dipalmitoyl-sn-glycero-3-hydroxyphosphoryloxy)-ethylamine.

The effectiveness of an adjuvant may be determined by measuring the induction of antibodies directed against an immunogenic polypeptide containing an egg surface protein polypeptide epitope, the antibodies resulting from administration of this polypeptide in vaccines which are also comprised of the various adjuvants.

The polypeptides may be formulated into the vaccine as neutral or salt forms. Pharmaceutically acceptable salts include the acid addition salts (formed with free amino groups of the peptide) and which are formed with inorganic acids, such as, for example, hydrochloric or phosphoric acids, or organic acids such as acetic, oxalic, tartaric, maleic, and the like. Salts formed with free carboxyl groups may also be derived from inorganic bases, such as, for example, sodium potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine and the like.

The vaccines of the invention may be multivalent or univalent. Multivalent vaccines are made from recombinant viruses that direct the expression of more than one antigen.

The patient to which the vaccine is administered is preferably a mammal, most preferably a human, but can also be a non-human animal including but not limited to cows, horses, sheep, pigs, fowl (*e.g.*, chickens), goats, cats, dogs, hamsters, mice and rats.

The vaccine formulations of the invention comprise an effective immunizing amount of the egg surface protein and a pharmaceutically acceptable carrier or excipient. Vaccine preparations comprise an effective immunizing amount of one or more antigens and a pharmaceutically acceptable carrier or excipient. Pharmaceutically acceptable carriers are well known in the art and include but are not limited to saline, buffered saline, dextrose, water, glycerol, sterile isotonic aqueous buffer, and combinations thereof. One example of such an acceptable carrier is a physiologically balanced culture medium containing one or more stabilizing agents such as stabilized, hydrolyzed proteins, lactose, etc. The carrier is preferably sterile. The formulation should suit the mode of administration.

The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. The composition can be a liquid solution, suspension, emulsion, tablet, pill, capsule, sustained release formulation, or powder. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, etc.

Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water free concentrate in a hermetically sealed container such as an ampoule or sachette indicating the quantity of active agent. Where the composition is administered by injection, an ampoule of sterile diluent can be provided so that the ingredients may be mixed prior to administration.

In a specific embodiment, a lyophilized egg surface protein polypeptide of the invention is provided in a first container; a second container comprises diluent comprising an aqueous solution of 50% glycerin, 0.25% phenol, and an antiseptic (*e.g.*, 0.005% brilliant green).

The precise dose of vaccine preparation to be employed in the formulation will also depend on the route of administration, and the nature of the patient, and should be decided according to the judgment of the practitioner and each patient's circumstances according to standard clinical techniques. An effective immunizing amount is that amount sufficient to produce an immune response to the antigen in the host to which the vaccine preparation is administered.

Use of purified antigens as vaccine preparations can be carried out by standard methods. For example, the purified protein(s) should be adjusted to an appropriate concentration, formulated with any suitable vaccine adjuvant and packaged for use.

Suitable adjuvants may include, but are not limited to: mineral gels, *e.g.*, aluminum hydroxide; surface active substances such as lysolecithin, pluronic polyols; polyanions; peptides; oil emulsions; alum, and MDP. The immunogen may also be incorporated into liposomes, or conjugated to polysaccharides and/or other polymers for use in a vaccine formulation. In instances where the recombinant antigen is a hapten, *i.e.*, a molecule that is antigenic in that it can react selectively with cognate antibodies, but not immunogenic in that it cannot elicit an immune response, the hapten may be covalently bound to a carrier or immunogenic molecule; for instance, a large protein such as serum albumin will confer immunogenicity to the hapten coupled to it. The hapten-carrier may be formulated for use as a vaccine.

Effective doses (immunizing amounts) of the vaccines of the invention may also be extrapolated from dose-response curves derived from animal model test systems.

The invention also provides a pharmaceutical pack or kit comprising one or more containers comprising one or more of the ingredients of the vaccine formulations of the invention. Associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

The present invention thus provides a method of immunizing an animal, comprising administering to the animal an effective immunizing dose of a vaccine of the present invention.

5.7.1.3 Packaging

The compositions may, if desired, be presented in a pack or dispenser device which may contain one or more unit dosage forms containing the active ingredient. The pack may for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration. Compositions comprising a compound of the invention formulated in a compatible pharmaceutical carrier may also be prepared, placed in an appropriate container, and labelled for treatment of an indicated condition.

5.7.2 Use of Antibodies Generated by Vaccines of the Invention

The antibodies generated against the antigen by immunization with the egg surface proteins of the present invention also have potential uses in vaccination against fertilization, sterilization, diagnostic immunoassays, passive immunotherapy, and generation of antiidiotypic antibodies.

The generated antibodies may be isolated by standard techniques known in the art (*e.g.*, immunoaffinity chromatography, centrifugation, precipitation, etc.) and used in diagnostic immunoassays. The antibodies may also be used to monitor treatment and/or disease progression. Any immunoassay system known in the art, such as those listed *supra*, may be used for this purpose including but not limited to competitive and noncompetitive assay systems using techniques such as radioimmunoassays, ELISA (enzyme-linked immunosorbent assays), "sandwich" immunoassays, precipitin reactions, gel diffusion precipitin reactions, immunodiffusion assays, agglutination assays, complement-fixation assays, immunoradiometric assays, fluorescent immunoassays, protein A immunoassays and immunoelectrophoresis assays, to name but a few.

The vaccine formulations of the present invention can also be used to produce antibodies for use in passive immunotherapy, in which short-term protection of a host is achieved by the administration of pre-formed antibody directed against a heterologous organism.

The antibodies generated by the vaccine formulations of the present invention can also be used in the production of antiidiotypic antibody. The antiidiotypic antibody can then in turn be used for immunization, in order to produce a subpopulation of antibodies that bind the initial antigen of the pathogenic microorganism (Jerne, 1974, Ann. Immunol. (Paris) 125c:373; Jerne, et al., 1982, EMBO J. 1:234).

In immunization procedures, the amount of immunogen to be used and the immunization schedule will be determined by a physician skilled in the art and will be administered by reference to the immune response and antibody titers of the subject.

6. EXAMPLE: 70kDa and 35-45 kDa Egg Surface Protein Clusters Involved in Sperm-Oolemma Binding and Fusion

The experiments presented in the following Example demonstrate the successful identification and characterization of glycosylated phosphatidylinositol (GPI)-anchored egg surface proteins required for fertilization. The results presented herein show that glycosyl-phosphatidylinositol (GPI)-anchored egg surface proteins are required for sperm to bind to and fuse with the egg. Thus, while treatment of mouse sperm with PI-PLC had no significant effect on either sperm-zona pellucida binding or sperm-egg binding and fusion, treatment of zona-intact or zona-free oocytes with PI-PLC blocked fertilization. The GPI-anchored egg surface proteins were characterized by 2-dimensional avidin blotting. As demonstrated herein, biotinylated mouse oocytes released protein clusters of approximately 70 kDa (pI 5) and 35-45 kDa (pI 5.5) following PI-PLC treatment.

6.1 INTRODUCTION

The role of GPI-anchored proteins in gamete interaction has yet to be thoroughly investigated. Mouse sperm surface hyaluronidase (also known as PH-20) is GPI-anchored and is thought to aid sperm in passage through the cumulus oopherous and possibly the zona pellucida by hydrolyzing the extracellular matrix protein, hyaluronic acid (Gmachl and Kreil, 1993, Proc. Nat. Acad. Sci. USA 90: 3569-3573; Myles and Primakoff, 1997, Biol. Reprod., 56: 320-327). Sperm agglutination antigen-1 (SAGA-1) is another sperm surface protein which has been shown to be a GPI-linked. While its role in fertilization has yet to be elucidated, in vitro assays have demonstrated that anti-SAGA-1 monoclonal antibodies agglutinate human sperm (Diekman et al., 1997, Biol. Reprod. 57: 1136-1144).

The effect of phosphatidylinositol-specific phospholipase C (PI-PLC) on mouse sperm-egg interaction was investigated in this study to determine if GPI-anchored proteins are involved in mammalian fertilization. In initial studies, mouse fertilization was blocked when sperm and zona-intact eggs were pre-treated for 30 min with a highly purified preparation of recombinant PI-PLC prior to gamete co-incubation. The stage in the fertilization cascade at which PI-PLC exerted its inhibitory effect, and whether one or both gametes were affected by PI-PLC was then investigated. Upon finding that PI-PLC treatment of zona-free oocytes, but not sperm, blocked sperm-egg binding and fusion, the released oolemmal GPI-anchored protein(s) were characterized using two-dimensional (2D) gel electrophoresis and cell surface labeling.

6.2 MATERIALS AND METHODS

PI-PLC Preparation

Recombinant PI-PLC was isolated from cultured supernatants of *Bacillus subtilis* (BG2320) transfected with the PI-PLC gene from *B. thuringiensis* (Henner et al., 1988, Nucl. Acid. Res. 16: 10383-10383). For details on PI-PLC purification see Low et al. (1988, J. Imm. Meth. 113: 101-111).

Gamete preparation

Preparation of mouse gametes for IVF was carried out essentially as described by (Bleil, 1991, Meth. Enz. 225: 253-263). Incubation of mouse gametes was performed in microdrops under paraffin oil at 37^{0}C and 5% CO_2 in Medium 199 (M199, Gibco, Grand Island, NY) supplemented with 4 mg/ml bovine serum albumin (BSA, Sigma, St. Louis, MO) and 3.5 mM sodium pyruvate (Gibco). Epididymides were collected from

sacrificed ICR strain retired breeders, placed in 250 µl medium, minced with watchmaker forceps, and sperm were allowed to swim out from the epididymides for 15 min. The sperm suspension was then placed under 1.5 ml medium and sperm were allowed to swim up for at least 1 h. Cumulus-oocyte complexes were collected from superovulated ICR strain females in M199. Cumulus cells were removed by treating eggs for 3 min with 1 mg/ml hyaluronidase (Sigma) in M199 and the eggs were then washed in six consecutive 50µl drops of M199. Following cumulus cell removal, oocytes were washed by passing the eggs through 50 µl drops of media covered with mineral oil using a pulled, heat polished, Pasteur pipette (employed in all experiments).

Sperm-zona binding and fertilization of zona-intact oocytes following pre-treatment of sperm and eggs with PI-PLC

Swim up sperm (approximately $3x10^6$ sperm/ml) were treated for 30 min with either 1U/ml PI-PLC or 1U/ml of heat inactivated (95°C for 5 min) PI-PLC in 100 µl of M199 under oil. Zona-intact oocytes (approximately 30 eggs per group) were treated for 30 min with either 1U/ml PI-PLC or 1U/ml inactivated PI-PLC in 100 µl of M199 under oil. Treated sperm were then added to the incubation drops containing treated eggs with a final droplet volume of 100 µl and a final sperm concentration of $1x10^6$ sperm/ml. Thus, PI-PLC was present in the incubation droplet during gamete interaction.

For the sperm-zona binding assay, the gametes were co-incubated for 1 h, washed gently 3 times in M199, and fixed in PBS containing 4% paraformaldehyde for 1 h. To quantitate binding, the oocytes were placed in a phosphate buffered saline (PBS)/4% paraformaldehyde solution between a microscope slide and an elevated cover slip. The oocytes were visualized at 200X using a light microscope (Zeiss Axioplan) and a single focal plane for each oocyte was selected in which the widest diameter of the zona pellucida could be visualized. The number of bound sperm in that focal plane was then determined.

To study fertilization of zona-intact oocytes, sperm were added to oocytes, the gametes were co-cultured for 2 h, washed 3 times in M199 to remove the PI-PLC and supernumerary sperm and incubated overnight at 37°C and 5% CO_2. To visualize sperm located within the perivitelline space following overnight incubation, gametes were incubated in 1 µM Hoechst dye #33342 (Sigma) for 10 min and washed 6 times in M199. The zygotes were then viewed at 200X as described above and zygotes with two blastomeres were scored as fertilized while one-celled oocytes were scored as unfertilized.

Fertilization of zona-intact oocytes which had been pre-treated with PI-PLC and washed prior to incubation with untreated sperm

Zona-intact oocytes (approximately 20 eggs per group) were treated for 30 min with either 1U/ml PI-PLC or 1U/ml inactivated PI-PLC in 100 µl of M199 under oil. The oocytes were then washed through six drops of M199. Untreated sperm were then added to the incubation drops containing treated eggs at a final volume of 100 µl and sperm concentration of $1X10^6$ sperm/ml. The gametes were then co-cultured for 2 h, washed 3 times in M199 and incubated overnight at 37°C and 5% CO_2. Zygotes with two blastomeres were scored as fertilized while one-celled oocytes were scored as unfertilized.

Sperm-oolemma binding and fusion following pre-treatment of either sperm or zona-free eggs with PI-PLC

Zonae pellucida were loosened by treating eggs with M199 containing 10 µg/ml chymotrypsin (Sigma) for 1 min. The eggs were then washed 6 times in M199 and loosened zonae were removed by mechanical agitation using a pulled Pasteur pipette. The eggs were allowed to recover from chymotrypsin treatment for 4 h in M199. The oocytes (approximately 15 oocytes per group) were then pre-loaded with 1 µM Hoechst dye #33342 (Sigma) for 10 min and washed 6 times in M199. Swim up sperm were collected and prepared as described in the gamete preparation section and allowed to capacitate for four hours during the oocyte recovery period. To evaluate the effect of treating only sperm with PI-PLC on subsequent sperm-egg binding and fusion, swim up sperm (approximately $3X10^6$ sperm/ml) were treated for 30 min with either 1U/ml PI-PLC or 1U/ml of heat inactivated PI-PLC in 100 µl of M199 under oil. The sperm were then washed 2 times by centrifugation (five min at 1000 g) in five ml volumes of M199 in 15 ml centrifuge tubes to remove the PI-PLC. The treated sperm were then added to untreated eggs (approximately 15 eggs per group) at a concentration of $1x10^5$ sperm/ml and the gametes were co-incubated for 40 min in 20 µl drops followed by gentle washing.

To evaluate the effect of treating only oocytes with PI-PLC on subsequent sperm-egg binding and fusion, the eggs were treated with either 1U/ml PI-PLC or heat inactivated PI-PLC for 30 min in 50 µl drops of M199 and washed through six 50 µl drops of M199 to remove the PI-PLC. The treated eggs were then incubated with untreated sperm ($1x10^5$ sperm/ml) for 40 min in 20 µl drops containing approximately fifteen oocytes per group followed by gentle washing of the eggs in M199. To determine if the effect of PI-PLC on zona-free oocytes was dose-dependent, oocytes were treated with either 5U/ml heat inactivated PI-PLC or increasing concentrations of PI-PLC (0-5U), washed, and incubated with untreated sperm as described above. To evaluate sperm-egg binding and fusion, the eggs from each group were then placed in M199 between a microscope slide and an elevated cover slip and visualized at 200X. Binding to the egg was scored by counting the total number of sperm bound per oocyte using phase contrast. Fusion with the egg was

scored by counting the number of decondensed sperm heads within each oocyte using fluorescent microscopy.

Artificial activation of oocytes following PI-PLC treatment

In order to ensure that PI-PLC treated eggs remained viable following PI-PLC treatment, zona-free eggs were treated with either 1U/ml PI-PLC or 1U/ml heat inactivated PI-PLC for 30 min as described in the sperm-oolemma binding and fusion assay. Immediately prior to egg activation, a small sample of oocytes were visualized using fluorescent microscopy to ensure that the main pool of oocytes were in metaphase II arrest following PI-PLC treatment. The remaining oocytes were activated by placing the eggs in 0.5 μM calcium ionophore (A23187, Sigma) for 5 min followed by three washes. The eggs were incubated for 40 min and oocytes were observed as described above. The eggs were considered activated if they had advanced from metaphase II arrest to anaphase II or telophase II (with second polar body).

Binding of beads coated with anti-integrin antibodies to eggs following PI-PLC treatment

Two-tenths μm yellow-green sulfate-derivatized latex beads (Molecular Probes, Eugene, OR) were coated with the goat (Go) H3 mAb (IgG2a,) to the α6 integrin subunit or an irrelevant control mAb (anti-MLV) of the same isotype as follows: beads from 10 μl of a 2% bead suspension were incubated with 10 μl of antibody (0.4 mg/ml) for 3 h at 4°C on an orbital platform mixer (Clay Adams, Parsippany, NJ). Beads were then washed twice with PBS, quenched for 1 h with 0.2 mg/ml goat anti-rabbit IgG (Sigma), washed twice with PBS, and then resuspended to 0.2% in PBS. Beads were used on the day of preparation and were sonicated 3 times for 5 sec each at 4° C immediately prior to use. Next, 20-40 zona-free eggs were either sham treated or treated with 1U/ml PI-PLC for 30 min, washed, and the protein-coated fluorescent beads (final concentration 0.02%) were added to each group. The eggs were incubated in a 5% CO_2 incubator and gently agitated every 15 min. After 1 h at 37°C, the eggs were washed through three 100 μl drops of fresh medium using an ~100 μm glass pipette. Following washes the eggs were placed 24-well dishes in small drops and overlaid with light mineral oil for imaging by confocal microscopy.

Two-Dimensional Gel Electrophoresis

Cumulus-oocyte complexes were collected and cumulus cells were removed as described above. To facilitate the collection of large numbers of zona-free oocytes for optimization of the 2D gel electrophoresis experiments, acid Tyrodes was used to removed

the zona pellucidae instead of chymotrypsin. To ensure that the acid Tyrodes method of zona removal did not affect the experimental outcome, the experiment was also performed using oocytes which had their zona removed using the chymotrypsin treatment method described in the sperm-oolemma binding and fusion section and the results were compared. For removal of zonae using acid Tyrodes, oocytes were placed in acid Tyrodes for 15 sec followed immediately by four washes in M199 (Evans et al., 1997, Dev. Biol. 187:79-93). The eggs were then allowed to recover for 4 h at 37°C and 5% CO_2. The eggs were then washed six times in BWW media (Irvine Scientific, Santa Ana, CA) containing 100 µg/ml polyvinylalcohol (PVA, Sigma), biotinylated with 2 µg/ml Sulfo-NHS biotin (Pierce, Rockford, IL) in BWW/PVA for 7 min at room temperature, and washed six times in BWW/PVA. The eggs were then split into two groups of 100 and either mock treated or treated with 1U/ml PI-PLC in 20 µl drops for 30 min. The supernatants were removed, the eggs were washed six times, and the oocytes and the oocyte supernatants were then frozen at -70°C in BWW/PVA containing protease inhibitors (Complete™, Boehringer Mannheim, Mannheim, Germany). The oocytes and supernatants were extracted in Celis lysis buffer containing 2% (v:v) NP-40, 9.8M urea, 100mM dithiothreitol (DTT), 2% ampholines (pH 3.5-10), and protease inhibitors for 30 min at room temperature (Rasmussen et al., 1991, Electrophoresis 12: 873-882). Isoelectric focusing (IEF) was performed using the Mini-PROTEAN II tube cell (Bio RAD, Richmond, CA) apparatus and protocol with an ampholine mixture (Pharmacia Biotech, Uppsala, Sweden) of pH 3.5-5 (30%), 3.5-10 (40%), 5-7 (20%), and 7-9 (10%). The tube gels were placed on 12 % mini slab gels and the focused proteins were separated in the second dimension at 20 mA per gel. The proteins were then electroblotted to nitrocellulose membranes at 125 mA for 45 min. The nitrocellulose membranes were blocked in PBS with 0.1% Tween and 5% dried milk for 30 min at room temperature, washed 1 time in PBS/0.1% Tween, and probed with 20 µg/ml strepavidin-HRP (Pierce) for 30 min at room temperature. The blots were washed 3 times 15 min in PBS/0.1% Tween (10 min per wash) and developed using enhanced chemiluminescence (Amersham Corp, Buckinghamshire, U.K.) for 5 min. As a control for determining the charge, mass, and location of the PI-PLC enzyme, one unit (1µg) of the PI-PLC preparation was mixed with Celis buffer and separated on a two-dimensional electrophoretic gel as described above and silver stained according to (Hochstrasser et al., 1988, Anal. Biochem. 173: 424-435).

Statistical Analysis

All in vitro assays were repeated at least three times. Experimental and control group averages were reported as means +/- the standard deviation. Groups were

compared using the students T test and differences were reported at the 0.05 level of significance.

6.3 RESULTS

In Vitro Assays

Co-culture of mouse sperm and zona-intact eggs in the presence of PI-PLC blocked fertilization

To determine the effects of PI-PLC on sperm-zona pellucida binding, mouse sperm and eggs were pre-treated separately with either 1U/ml heat inactivated PI-PLC or 1U/ml PI-PLC for 30 min, and co-cultured in the presence of PI-PLC. Following one hour of co-culture, sperm were observed to bind abundantly to the zona of both control (Fig. 1A) and treated gametes (Fig. 1B). Quantitation revealed no significant difference in the number of sperm-bound per zona pellucida between the control group (43.9 sperm/zona) and the PI-PLC treated group (45.9 sperm/zona) (Fig. 1E).

To determine the effects of PI-PLC on fertilization of zona-intact oocytes, sperm and eggs were pre-treated separately with PI-PLC as described above, co-cultured in the presence of PI-PLC for two hours to permit fertilization, washed 3 times to remove the PI-PLC and supernumerary sperm, and incubated overnight. It is noteworthy that the molecular weight of PI-PLC is 30-34 kDa and the zona pellucida is freely permeable to molecules having a mass of less than 170 kDa (Legge, 1995, J. Exp. Zool. 271: 145-150); therefore the oolemma was likely exposed to PI-PLC during treatment. Twenty four hours following gamete co-culture, the majority of eggs in the control group had undergone cleavage whereas few oocytes had done so in the PI-PLC treated group (Fig. 1C and 1D). Quantitation revealed a significant reduction in the fertilization rate from 59.6% in the inactivated PI-PLC control to 2.8% in the PI-PLC treated group (Fig. 1E). A most striking observation was the large number of sperm which accumulated within the perivitelline space of uncleaved PI-PLC treated eggs (Fig. 1D, inset) when compared to controls (Fig. 1C, inset).

PI-PLC effect on fertilization of zona-intact eggs

The fertilization experiments utilizing zona-intact oocytes demonstrated that pre-treatment of sperm and eggs with PI-PLC followed by gamete co-incubation in the presence of PI-PLC inhibited fertilization while sperm-zona binding was unaffected. It then became of interest to determine whether the fertilization inhibition of zona-intact eggs by PI-PLC was due to an effect on the sperm or the egg. Therefore, either sperm or eggs which

had been treated with PI-PLC and washed free of enzyme prior to gamete co-incubation were used to attempt to fertilize zona-intact oocytes. When zona-intact oocytes were treated with 1U/ml PI-PLC for 30 min and washed prior to incubation with untreated sperm, fertilization rates were reduced from 63% in the control group to 3% in the PI-PLC treatment group. This reduction in fertilization is similar to that observed when PI-PLC was present in the fertilization medium during gamete interaction. This result suggested that PI-PLC blocked fertilization by affecting the oolemma.

In evaluating the effect of PI-PLC on sperm, both the control and treatment groups underwent a loss in motility following centrifugal washing and neither were able to fertilize zona-intact eggs. Therefore, it was not possible to establish if PI-PLC treatment affected sperm fertilizing ability using zona-intact eggs. The reduction in motility, however, did not affect sperm-oolemma binding and fusion, and the locus of PI-PLC action was refined using zona-free eggs.

Pre-treatment of zona-free oocytes with PI-PLC inhibits both sperm-egg binding and fusion

When sperm were treated with either 1U/ml heat inactivated PI-PLC or 1U/ml PI-PLC for 30 min, washed, and incubated with untreated zona-free oocytes, no statistical difference was observed in sperm-egg binding or fusion between the control (Fig. 2A) and treatment group (Fig. 2B). In the control group 5.4 sperm bound per egg whereas 8.0 sperm bound per egg in the PI-PLC treated group (Fig. 2E). Similarly, when sperm were treated with PI-PLC, there was no significant difference in sperm-egg fusion when comparing the control group (1.7 sperm fused per egg) to the treatment group (1.5 sperm fused per egg). By contrast, when zona-free oocytes were treated with either 1U/ml heat inactivated PI-PLC or 1U/ml PI-PLC for 30 min, washed, and incubated with untreated sperm, a significant decrease in binding and fusion was observed in the treatment group (Fig. 2D) when compared to controls (Fig. 2C). In the control group 6.2 sperm bound per egg and 1.9 sperm fused per egg compared to the PI-PLC treated group where 2.1 sperm bound per egg and 0.02 sperm fused per egg (Fig. 2E). These results further support our results that the PI-PLC effect on fertilization is mediated at the oolemma. Of interest was the observation that while most of the PI-PLC treated oocytes had only 0 to 2 bound sperm, a small percent of the treated eggs were bound by numerous sperm yet fusion was still blocked (see oocyte indicated by arrow in Fig. 2D).

Treatment of zona-free oocytes with PI-PLC inhibits sperm-egg binding and fusion in a dose-dependent manner

Oocytes were treated with either 5U/ml of heat inactivated PI-PLC, no PI-PLC, or increasing amounts of PI-PLC (0, 0.05, 0.1, 0.5, 1, 5U/ml), washed, and incubated with untreated sperm to determine if the inhibitory effect of PI-PLC on the oolemma was dose-dependent. Results showed that, as the concentration of PI-PLC was increased, sperm-egg binding and fusion rates decreased in a dose-dependent manner (Fig. 3). The maximal inhibitory effect on sperm-egg binding was reached at 5U/ml while the maximal inhibitory effect on fusion occurred at 1U/ml. The recommended dose for releasing most GPI-anchored proteins from intact cells using *B. thuringiensis*-derived PI-PLC is 1U/ml. The dose-dependent inhibition of both sperm-egg binding and fusion by PI-PLC supports the hypothesis that treating oocytes with PI-PLC releases GPI-anchored proteins from the oolemma which are required for fertilization.

Purity of the recombinant PI-PLC enzyme

To evaluate the purity of the PI-PLC preparation, an aliquot of the enzyme was separated by 2D electrophoresis and the gel was silver stained. Results showed that only one prominent protein spot (approximately MW 30 kDa, approximate pI 6) and several smaller protein spots immediately surrounding the prominent protein (possibly isoforms of PI-PLC) could be visualized (Fig. 4). The reported molecular weight of PI-PLC under reducing conditions is 30-35 kDa (Low and Saltiel, 1988, Science, 239: 268-275). These results indicate that the recombinant PI-PLC preparation used for the experiments in this study was highly purified.

Treatment of zona-free eggs with PI-PLC had no observable effect on artificial egg activation

Artificial activation of oocytes was performed to ensure that the oocytes remained viable following PI-PLC treatment. Zona-free eggs were either treated with 1U/ml heat inactivated PI-PLC or 1U/ml PI-PLC, washed, and samples of control and treated eggs were observed to ensure that the oocytes remained in meiotic arrest following PI-PLC treatment. All oocytes (14 oocytes per group) which were observed in both control (Fig 5A, inset) and treatment groups (Fig 5B, inset) remained in meiotic arrest. The remaining oocytes were then artificially activated with 0.5 µm calcium ionophore A23187 for 5 min, washed, and cultured for 40 min. Oocytes which had progressed from metaphase II arrest to anaphase II or telophase were scored as activated. Following culture, there was no observable difference in the number of eggs which resumed meiotic cell division in the control eggs (31 out of 47 oocytes activated, Fig. 5A) compared with PI-PLC treated eggs (27 out of 44 oocytes activated, Fig. 5B).

Treatment of zona-free oocytes with PI-PLC does not effect the ability of beads coated with anti-α6β1 antibodies to bind to oocytes

A bead binding experiment was performed to determine if PI-PLC treatment of zona-free mouse oocytes affected the antibody recognition of a well characterized egg surface integrin. Fluorescent beads were coated with α6β1 antibodies and incubated with either untreated or PI-PLC treated oocytes. No difference was observed in the number of α6β1 antibody-coated beads bound per oocyte between the control (Fig. 6A) and treatment group (Fig. 6B). Minimal bead binding was observed when beads were coated an with equivalent concentration of an irrelevant antibody and incubated with untreated eggs (Fig. 6C). These results indicate that treatment of oocytes with PI-PLC does not affect the ability of anti-α6β1 antibodies to bind its cognate antigen on the oolemmal. Therefore, using the egg surface integrin α6β1 as a model protein, the interactions of a non-GPI-anchored protein are not compromised by treatment with the PI-PLC preparation. This finding supports the hypothesis that the inhibitory effect of PI-PLC on fertilization was specifically due to the release of one or more GPI-anchored proteins from the oolemma following PI-PLC treatment and not to a non-specific perturbation of molecules on the surface by either PI-PLC or a contaminating substance in the enzyme preparation.

Treatment of Biotinylated Oocytes with PI-PLC Releases GPI-Anchored Proteins

Results from the in vitro assays led to the hypothesis that treatment of the oolemma with PI-PLC released one or more functionally relevant GPI-anchored protein(s) from the mammalian egg surface. Two-dimensional gel electrophoresis was then utilized to determine if the putative PI-PLC releasable proteins could be resolved and visualized. For these experiments 200 zona-free mouse oocytes were biotinylated, washed six times, separated into two groups, and incubated with or without 1U/ml PI-PLC for 30 min. Following six washes, egg proteins were extracted. The supernatants from the treated eggs as well as the egg extracts were separated by 2D electrophoresis, electroblotted to nitrocellulose membranes, probed with strepavidin-HRP, and biotinylated proteins were visualized on radiograms using enhanced chemi-luminescence. The 2D gel repertoire of biotin-labeled egg surface proteins present in the extracts of control eggs is presented in Fig. 7A. Approximately 20 biotin labeled proteins were resolved ranging in molecular weight from approximately 35 to 120 kDA with isoelectric points from 4.5 to 5.5. Following PI-PLC treatment of the oocytes (Fig. 7C), a decrease in the presence of the 70 kDa (pI 5) and 35-45 kDa (pI 5.5) protein clusters indicated by arrows in Fig. 7A was observed in extracts of eggs. The three spots denoted by asterisks in Fig. 7A represent proteins that bound strepavidin-HRP non-specifically and were detected on 2D blots of oocytes which were not biotinylated. 2D gel analysis of supernatant collected from untreated oocytes following 30

min of incubation showed that no biotinylated egg surface proteins were released into the medium (Fig. 7B). The repertoire of biotin-labeled proteins remaining on the egg surface following PI-PLC treatment is shown in Fig. 7C. Arrows denote the 70 kDa (pI 5) and 35-45 kDa (pI 5.5) protein clusters which were prominent in the extracts of untreated eggs (Fig. 7A) but decline in intensity in the extracts of eggs treated with PI-PLC. In contrast to the supernatant from untreated eggs (Fig. 7B), the supernatant from eggs treated with PI-PLC (arrows, Fig. 7D) revealed prominent 70 kDa and 35-45 kDa protein clusters of similar molecular weights and isoelectric points to those released from the eggs surface following PI-PLC treatment (Fig. 7C). It is likely that these GPI-anchored egg surface proteins are required for sperm-egg binding and fusion. Two minor protein spots at approximately 75-78 kDA and pI 5.5 (arrowheads, Fig. 7D) were also released from the egg surface into the supernatant following PI-PLC treatment. However, these proteins were only seen in two of five replications of this experiment.

Acid Tyrodes was used to remove the zona pellucida prior to cell surface biotinylation in the majority of the blotting experiments while chymotrypsin was used to de-zonulate oocytes for the sperm-egg binding and fusion assays. To ensure that the method of zona removal did not alter the pattern of labeled egg surface and PI-PLC released proteins, chymotrypisin was also used to de-zonulate oocytes prior to surface labeling and avidin blotting. Other than substituting chymotrypsin to de-zonulate oocytes, all conditions for this experiment were identical to the previous experiment using acid Tyrodes. The repertoire of surface labeled proteins present in the extracts of control eggs is shown in Fig. 8A. As with the previous experiment employing acid Tyrodes, there were 70 kDa (pI 5) and 35-45 kDa (pI 5.5) protein clusters present in the extracts of eggs which were not treated with PI-PLC (denoted by arrows in Fig. 8A). These proteins declined in intensity in the extracts of eggs treated with PI-PLC (denoted by arrows in Fig. 8C). Protein clusters of similar masses and isoelectric points to those which declined in intensity following PI-PLC treatment were observed in the supernatants from PI-PLC treated oocytes (denoted by arrows in Fig. 8D). No surface labeled proteins were seen in the supernatant from oocytes which were not treated with PI-PLC (Fig. 8B). Thus, PI-PLC releases 70 kDa and 35-45 kDa protein clusters regardless of the method of zona removal increasing confidence in the reproducibility of our findings.

6.4 DISCUSSION

The Effect of PI-PLC on Sperm-Egg Binding and Fusion is Mediated at the Level of the Oolemma

The results of the fertilization studies using zona-intact oocytes demonstrated that fertilization in vitro is blocked when mouse sperm and zona-intact oocytes are pre-

treated with PI-PLC prior to gamete co-culture in the presence of PI-PLC. The effect of the enzyme on fertilization does not appear to be at the level of sperm-zona pellucida interaction because PI-PLC had no significant effect on sperm-zona binding and significant numbers of perivitelline sperm were observed in the PI-PLC treated group following overnight incubation. These results suggest that GPI-linked sperm surface proteins may not be essential for penetration of the zona pellucida in mice. Another important finding from the zona-intact fertilization studies was that when zona-intact oocytes are treated with PI-PLC and washed prior to incubation with untreated sperm, fertilization is blocked. This result implies that the effect of PI-PLC is mediated at the oolemma. We then used the zona-free sperm-egg binding and fusion assay to further establish which gamete was being affected by PI-PLC treatment. Results showed that PI-PLC treated and washed sperm could bind to and fuse with untreated zona-free oocytes at levels similar to controls. Therefore, the effect of PI-PLC on fertilization does not appear to be mediated at the level of the sperm surface. Previous investigators have treated mouse sperm with similar PI-PLC concentrations and found that numerous iodinated GPI-anchored proteins could be visualized on 1D gel autoradiographs of supernatants from treated sperm (Thaler and Cardullo, 1995, Biochem. 34, 7788-7795), therefore, the lack of effect seen in this study does not appear to be due to and insufficient enzyme concentration. Perhaps the most important observation of this study was that fertilization was blocked when either zona-intact or zona-free eggs were treated with PI-PLC and washed prior to incubation with untreated sperm. These results imply that one or more GPI-anchored oolemmal proteins are required for sperm-egg binding and fusion.

The Inhibitory Effect of PI-PLC on Fertilization is Specific to GPI-Anchored Proteins

As noted in the introduction several investigators have previously studied the effects of various preparations of phospholipase C (PLC) on fertilization. Most PLC has a broad specificity for a variety of phospholipids with only minor differences in efficiency of hydrolysis (Low et al., 1986, Biochem. J. 237: 139-145). Several phospholipases have additional specificity for particular phospholipid structures. The *B. thuringiensis*-derived recombinant PI-PLC is specific for phosphatidylinositol and glycosylated phosphatidylinositol phospholipids (Low et al., 1988, J. Imm. Meth. 113: 101-111) and for this reason was selected for investigating the role of GPI-anchored proteins in sperm-egg interaction in this study.

Due to the broad specificity of PLC, results from previous studies investigating the inhibitory effects of PLC on sperm-egg interaction (Hirao and Yanagimachi, 1978, Gam. Res. 1: 3-12; Boldt et al.,1988, Biol. Reprod. 39: 19-27) do not

directly address the question of whether oolemmal GPI-anchored proteins are involved in fertilization. Also, other investigators found that while relatively impure PLC preparations blocked sperm-egg fusion, 'purer' PLC preparations (including PI-PLC) did not affect sperm-egg fusion (Clark and Koehler, 1988, Gam. Res. 19: 339-348). The authors concluded that contaminants in the 'impure' PLC preparations disrupted oocyte morphology (as determined by electron microscopy) which in turn impaired the ability of sperm to fuse with the egg. Regarding the effects of treating oocytes with PI-PLC, the authors did report a slight, but significant, inhibition of sperm-egg fusion. In the (Clark and Koehler, 1988, Gam. Res. 19: 339-348) experiments, short treatment times (3 min) were used for both PLC and PI-PLC because of the disruptive effect of impure PLC preparations on the oocytes. However, while it is known that PLC can cause non-specific membrane perturbations in intact cells (Mollby et al., 1973, Toxicology 11: 139-147), the same phenomenon has not been reported for PI-PLC. Therefore, it is possible that longer treatment of hamster oocytes with PI-PLC might have generated a greater inhibitory effect on sperm-egg fusion. In fact, we have recently found that when zona-free hamster oocytes are treated for 30 min with 1U/ml of PI-PLC, washed, and incubated with untreated human sperm, binding and fusion is almost completely blocked. However, when human sperm are treated with PI-PLC, washed, and incubated with untreated zona-free hamster oocytes, binding is significantly enhanced, while fusion is not affected. The GPI-anchored proteins which are released from the hamster oolemma following PI-PLC treatment have also been visualized on 2D avidin blots (see Example in Section 7).

In the present study, a number of controls were performed to validate that the effect of PI-PLC on mouse oocytes was specifically due to the release of GPI-anchored proteins following PI-PLC treatment. When one μg of the PI-PLC preparation was separated by 2D electrophoresis and silver stained, only one prominent protein spot (~30 kDa) could be visualized. This spot corresponded to the anticipated mass of PI-PLC. To ensure that the effect of the enzyme on fertilization was not due to an inorganic component of the enzyme preparation, heat inactivated PI-PLC was used as a control for all in vitro assays. Further, the effect of PI-PLC on fertilization was dose-dependent. The dose-response curve trial indicated that the maximal effect of PI-PLC on sperm-egg binding and fusion was 1-5 U per ml; which corresponds to that which is recommended for maximal release of GPI-anchored proteins (Dr. Martin Low, personal communication). Importantly, oocytes which had been treated with PI-PLC could be artificially activated with calcium ionophore, indicating that treated oocytes remained functionally viable. Finally, beads coated with $\alpha 6\beta 1$ antibodies could bind to PI-PLC treated eggs in a manner similar to that of controls, thus indicating that non-GPI-linked oolemmal proteins did not appear to be

affected by the PI-PLC preparation. Taken together, the in vitro data demonstrate that one or more oolemmal GPI-anchored protein(s) are required for fertilization and release of these proteins from the oolemma following PI-PLC treatment prevents oocytes from being fertilized by sperm.

Resolution of the Repertoire of Mammalian Oolemmal Surface Proteins Using Two-Dimensional Gel Electrophoresis and Cell Surface Vectorial Labeling

Although several investigators have surface labeled oocytes and visualized the proteins on 1D blots, there are no previous studies which utilize two-dimensional gel electrophoresis to visualize surface-labeled oolemmal proteins. Boldt et al. (1989, Gam. Res. 23: 91-101) radioiodinated zona-free mouse eggs and found that there were 8-10 egg surface proteins that incorporated iodine with major bands at 145-150, 94 and 23 kDa. (Flaherty and Swann, 1993, Mol. Reprod. Dev. 35: 285-292) evaluated the oolemmal protein pattern of biotinylated zona-free mouse eggs and found that there were two predominant bands of 82 and 69 kDa, eight major bands, and 14 minor bands which could be visualized. Recently, (Ya Zhong et al.,1997, Mol. Reprod. Dev. 47: 120-126) investigated the pattern of human, hamster and mouse oolemmal proteins after biotinylation and found that the overall staining pattern is similar between species. In human oocytes, these investigators identified 13 biotinlyated protein bands of which a 71kDa protein predominated. This study also observed the changes in the pattern of oolemmal protein expression during oocyte maturation. The total number of membrane proteins decrease from germinal vesicle to MII stage oocytes, while during this same time period, the relative proportion of the 71 kDa band increased from 9.9% to 27%.

In the present study, the repertoire of murine oolemmal surface proteins has, for the first time, been resolved using two-dimensional gel electrophoresis and cell surface biotinylation. Results from the 2D avidin blots (Fig. 7A) demonstrate that there appear to be a limited number (~ 20) of biotinylatable surface proteins on the mouse oolemma with a predominant protein at ~70 kDa (indicated by the top arrow in Fig. 7A). Further, this study has also indicated that the method of zona removal did not significantly alter the 2-D repertoire of oolemmal proteins. We found that, while there are some differences, the overall surface labeling patterns are similar when comparing acid Tyrodes de-zonulated oocytes (Fig. 7) with chymotrypsin de-zonulated oocytes (Fig. 8). The majority of surface-labeled proteins detected are acidic, with approximate isoelectric points ranging between 4.5 and 5.5. The resolution of this cohort of egg surface proteins by 2-D gel electrophoresis provides a basis for proceeding with the microsequencing, identification, and cloning of unknown proteins from oocytes.

Characterization of GPI-Anchored Proteins Using Two-Dimensional Gel Electrophoresis

The ability to visualize a repertoire of biotinylated oolemmal proteins on 2D avidin blots then allowed us to determine if treatment of biotin-labeled oocytes with PI-PLC releases oolemmal proteins from the egg surface into the supernatant. Comparison of PI-PLC treated and untreated eggs as well as the egg supernatant proteins from these groups reveal a predominant 70 kDa and less prominent 35-45 kDa protein cluster (indicated by arrows in Fig. 7A) to be released from the oolemmal surface into the supernatant following PI-PLC treatment (Fig.7C and Fig. 7D).

When analyzing the released protein(s) in (Fig. 7D) both the 70 kDa and the 35-45 kDa protein clusters appear as a series of closely aligned columns having slightly different isoelectric points. Previous literature on 2-D gel protein patterns (Shackelford et al., 1980, J. Exp. Med. 151: 144-165); (Negm et al., 1991, Comp. Biochem. Phys. Comp. Biochem. 99: 741-749) suggest that these protein clusters represent isoforms of a glycosylated protein. It is not known at this time whether the 70 kDa and 35-45 kDa protein clusters are in fact different proteins or are different isoforms of the same protein. While the 70 kDa and 35-45 kDa protein clusters represent candidate mediators of sperm-egg binding and fusion we have no direct evidence as yet they are, in fact, required for fertilization.

Possible Role of the GPI-Anchored Proteins in the Fertilization Cascade

The precise function of these PI-PLC sensitive proteins in the fertilization process is unknown at this time. One possibility is that the molecules are involved in the block to polyspermy as is the Ascidian GPI-anchored molecule, N-acetylglucosaminidase (Lambert, 1989, Development 105: 415-420). In Ascidians, sperm first bind to the ligand, N-acetylglucosamine, on the vitelline coat (VC), then penetrate through the VC and the perivitelline space to reach the egg surface, where sperm-egg fusion then occurs (Rosati and De Santis, 1980, Nature 283: 762-764). Ascidian eggs release N-acetylglucosaminidase from the cell surface into the sea water immediately following fertilization (Lambert, 1989, Development 105: 415-420). The enzyme can also be released by treating Ascidian eggs with exogenous PI-PLC (Lambert and Goode, 1992, Dev. Biol. 154: 95-100). Upon release, N-acetylglucosaminidase then modifies N-acetylgucosamine on the vitelline coat, thus preventing subsequent sperm penetrations. The investigators hypothesize that N-acetylglucosaminidase is released from the egg surface by the activity of endogenous phospholipases operating on the extracellular side of the surface (Goode et al., 1997, Dev. Growth. Diff. 39: 655-660).

In mice N-acetylglucosaminidase is also released from oocytes following fertilization (Miller et al., 1993, J. Cell. Biol. 123: 1431-140l) and subsequently occupies the sperm β1,4-Galactosyltransferase binding site on the zona pellucida, thus preventing polyspermy. While the molecular weight of mouse N-acetylglucosaminidase has yet to be determined, in Ascidians the released protein exhibits bands at 62 and 70 kDa (Lambert and Goode, 1992, Dev. Biol. 154: 95-100). It seems unlikely that the PI-PLC sensitive oolemmal protein in the present study is a GPI-anchored form of N-acetylglucosaminidase because sperm-zona binding was not affected by treating zona-intact eggs with PI-PLC. Further, N-acetylglucosaminidase does not appear to be GPI-anchored and has been localized to the cortical granules in mice (Miller et al., 1993, J. Cell. Biol. 123: 1431-140l) and therefore would not be susceptible to PI-PLC treatment.

Investigators have shown that a block to polyspermy also exists in mammals at the level of sperm-oolemma binding and fusion (Horvath et al.,1993, Mol. Reprod. Dev. 34: 65-72), however, the mechanism by which this block occurs is unknown. Results from this study demonstrate that when oocytes are treated with PI-PLC, fertilization is blocked and GPI-anchored protein(s) are released from the oolemma. One model to explain the observed results would posit that the GPI-anchored protein(s) performs a dual role as an oolemmal receptor for sperm and also mediates the oolemmal block to polyspermy. In this model when sperm bind to the GPI-anchored sperm receptor during the initial sperm-oolemmal interaction, second messenger pathways might then be activated which would result in the rapid release (possibly by endogenous PLC) of the remaining GPI-anchored sperm receptors from the oolemmal surface, thus preventing subsequent sperm from binding to the oolemma. If this hypothesis were correct, then treatment of oocytes with exogenous PI-PLC in this study could have blocked sperm-egg binding and fusion by releasing the GPI-anchored sperm receptors prior to sperm-egg interaction. Experiments are currently underway to isolate, characterize, and clone the released protein(s) and investigate their role in fertilization.

In conclusion, in vitro fertilization studies reported here demonstrate that the treatment of mouse sperm with PI-PLC does not affect the ability of sperm to penetrate the zona pellucida or to the ability of sperm to bind to and fuse with the oolemma. However, treatment of mouse oocytes with PI-PLC dramatically reduces the oocytes ability to bind to and fuse with sperm. The effect of PI-PLC on fertilization was: dose-dependent; PI-PLC did not alter the ability of treated eggs to be artificially activated; and did not effect the ability of beads coated with α6β1 integrin antibodies to bind the oolemma. 2D gel experiments demonstrate that 70 kDa (~ pI 5) and 35-45 kDa (~pI 5.5) protein clusters are

released from the oolemma following PI-PLC treatment. It seems likely that the released GPI-anchored protein(s) are required for fertilization.

7. EXAMPLE: Characterization of a PI-PLC-sensitive Oolemmal Protein Mediating Human Sperm-Hamster Egg Binding and Fusion

In the Example presented herein, the effects of phosphatidylinositol-specific phospholipase C (P1-PLC) on human sperm-hamster egg interaction were investigated to determine if glycosyiphosphatidylinositol (GPI) anchored proteins are involved in sperm-egg binding and fusion. Two-dimensional electrophoresis was used to visualize proteins released from hamster oocytes following P1-PLC treatment. For the binding and fusion assay, either sperm or eggs were treated with lU/mi PI-PLC for 30 min and washed prior to gamete co-incubation. Treatment of human sperm with PI-PLC significantly enhanced sperm-egg binding while having no effect on sperm-egg fusion. Treatment of zona-free hamster oocytes with P1-PLC blocked sperm-egg binding and fusion. In order to identify the oolemmal GPI-anchored proteins involved in fertilization, egg surface proteins were labeled with sulfo-NHS biotin and either mock treated or treated with PI-PLC. Egg protein extracts and egg supernatant proteins from each group were then analyzed by two-dimensional gel electrophoresis followed by avidin blotting. Comparison of blots demonstrated that a predominant biotinylated 25-40 kDa protein cluster (pI 5-6) apparent in the mock treated egg extract blot was absent in the PI-PLC treated egg extract blot. A protein cluster of identical molecular weight and isoelectric point as the predominant 25-40 kDa protein cluster was observed in the P1-PLC supernatant blot while no proteins could be seen in the control supernatant blot. These results demonstrate that treatment of hamster oocytes with PI-PLC inhibits sperm-egg interaction and releases a 25-40 kDa protein cluster (p1 5-6) from the oolemma. This released protein cluster represents an oolemmal GPI-linked surface protein(s) which is involved in human sperm-hamster egg interaction.

7.1 MATERIALS AND METHODS

PI-PLC Preparation

The phosphatidylinositol-specific phospholipase C preparation used for this study was purchased from Boehringer Mannheim (Indianapolis, IN). The PI-PLC was isolated from the cultured filtrate of *Bacillus cerus* and migrates as a single band on an SDS-PAGE gel at 29,000 Daltons.

Sperm Penetration Assay

Gamete Preparation - Gamete incubations were carried out in microdrops under paraffin oil at 37°C and 5% CO_2. Ejaculated human semen was allowed to liquefy for at least 30 min. Five hundred µl of the ejaculate was placed under 2 ml of BWW medium (Irvine Scientific, Santa Ana, CA) with 5 mg/ml human serum albumin (HSA, Sigma) for 30 min. and the sperm were allowed to swim up. The swim up sperm were then washed twice by centrifugation (8 min at 600 g) in 10 ml volumes of BWW in 15 ml centrifuge tubes. The sperm were capacitated overnight in 250 µl microdrops of BWW with 30 mg/ml HSA at a concentration of 20 x 10^6 sperm/ml. Cumulus-oocyte complexes were collected from superovulated Golden Syrian hamsters and placed in BWW with 5 mg/ml HSA. Cumulus cells were removed by treating eggs with 1 mg/ml hyaluronidase (Sigma) for 3 min. Oocytes were then washed (for this treatment and all subsequent treatments) by passing the eggs through 20 µl drops of media covered with mineral oil using a pulled, heat polished, Pasteur pipette. Zonae pellucida were removed by treating eggs with 1 mg/ml trypsin (Sigma) for 30 sec followed by 5 washes.

Treatment of human sperm with PI-PLC - Following overnight capacitation, 2 x 10^6 sperm were treated for 30 min. with either 1U/ml PI-PLC or 1U/ml of heat inactivated (95°C for 5 min) PI-PLC in 100 µl of BWW with 30 mg/ml HSA. The sperm were then washed twice by centrifugation in five ml volumes of BWW in 15 ml centrifuge tubes to remove the PI-PLC. Treated sperm were then added to untreated zona-free hamster oocytes (~12 per treatment group) at a concentration of 2 X 10^6 sperm/ml in 20 µl drops of BWW with 30 mg/ml HSA and the gametes were co-incubated for 3 h.

Treatment of zona-free hamster oocytes with PI-PLC - zona-free oocytes were treated for 30 min. with either 1U/ml PI-PLC or lU/ml of heat inactivated PI-PLC in 20 ml drops of BWW with 30 mg/ml HSA. The eggs were then washed through 5 microdrops and incubated with untreated human sperm at a concentration of 2 X 10^6 sperm/ml for 3 h.

Quantitation of sperm-egg binding and fusion - Following gamete co-incubation loosely bound sperm were removed from the oocytes by gentle pipetting. The eggs were then treated with 1mM acridine orange-3% in DMSO (Sigma) for 15 sec to stain the chromatin and washed through three 20 µl microdrops. To quantitate binding, the oocytes were placed between a microscope slide and an elevated cover slip, the oocytes were visualized at 200X using a light microscope (Zeiss Axioplan) and the number of sperm bound per oocyte was recorded. The number of sperm fused per egg was scored by counting the number of acridine orange-stained decondensed sperm heads within each oocyte using fluorescent microscopy.

Artificial activation of oocytes

In order to ensure that PI-PLC treated eggs remained viable following PI-PLC treatment, zona-free eggs were treated with either 1U/ml PI-PLC or 1U/ml heat inactivated PI-PLC for 30 min as described in the sperm penetration assay section. Following treatment, oocytes were preloaded with 1 μm Hoechst dye #33342 (Sigma) for 10 min to stain chromatin and washed three times. The oocytes were then activated by placing the eggs in 0.5 μM calcium ionophore A23187 (Sigma) for 5 min followed by three washes. The eggs were incubated for three hours and oocytes were observed as described in the sperm penetration assay section. The eggs were considered activated if they had advanced from metaphase II arrest to anaphase II or telophase II (with second polar body).

Two Dimensional Gel Electrophoresis

Hamster oocytes were collected and de-zonulated as described in the sperm penetration assay section. The zona-free eggs were then washed six times in BWW media containing 100 μg/ml polyvinylalcohol (PVA, Sigma), biotinylated with 2 mg/ml Sulfo-NUS biotin (Pierce, Rockford, IL) in BWW/PVA for 7 min at room temperature, and washed six times in BWW/PVA. The eggs were then split into two groups of 130 and either mock treated or treated with 1U/ml PI-PLC in 20 μl drops for 30 min. The supernatants were removed, the eggs were washed six times, and the oocytes and the oocyte supernatants were then frozen at -70°C in BWW/PVA containing protease inhibitors (Complete™, Boehringer Mannheim, Mannheim, Germany). The oocytes and supernatants were extracted in Celis lysis buffer containing 2% (v:v) NP-40, 9.8M urea, 100mM dithiothreitol (DTT), 2% ampholines (pH 3.5-10), and protease inhibitors for 30 min. at room temperature (Rasmussen et al., 1991, Electrophoresis 12: 873-882). Isoelectric focusing (IEF) was performed using the Mini-PROTEAN II tube cell (Bio RAD, Richmond, CA) apparatus and protocol with an ampholine mixture (Pharmacia Biotech, Uppsala, Sweden) of pH 3.5-5 (30%), 3.5-10 (40%), 5-7 (20%), and 7-9 (10%). The tube gels were placed on 12 % mini slab gels and the focused proteins were separated in the second dimension at 20 mA per gel. The proteins were then electroblotted to nitrocellulose membranes at 125 mA for 45 min. The membranes were then stained with Protogold for 10 min to visualize the egg proteins and washed briefly with water. Next, the membranes were blocked in PBS with 0.1% Tween and 5% dried milk for 30 min at room temperature, washed 1X in PBS/0.1% Tween, and probed with 20 μg/ml strepavidin-HRP (Pierce) for 30 min at room temperature. The blots were washed 3X in PBS/0.1% Tween (10 min per wash) and the biotinylated proteins were visualized using TMB as a substrate.

Statistical Analysis

The sperm penetration assay and egg activation assay were each repeated three times. Experimental and control group averages were reported as means +/- the standard deviation. Groups were compared using the students T test and differences were reported at the 0.05 level of significance.

7.2 RESULTS

Pre-treatment of sperm with PI-PLC significantly enhances human sperm-hamster egg binding while having no effect on sperm-egg fusion

When capacitated human sperm are treated with PI-PLC, washed and co-incubated with untreated zona-free hamster oocytes, there is a significant increase in the number of sperm bound to the oolemma (21.3 sperm per egg) when compared to the control group in which sperm were treated with heat inactivated PI-PLC (10.33 sperm per egg) (Fig. 8A). However, treatment of capacitated human sperm with PI-PLC, did not significantly effect sperm-egg fusion (1.7 sperm fused per egg) when compared to the control group (1.4 sperm per egg; Fig. 8B).

Pre-treatment of zona-free hamster oocytes with PI-PLC blocks human sperm-hamster egg binding and fusion

When zona-free hamster oocytes are treated with PI-PLC, washed and co-incubated with untreated capacitated human sperm, there is a significant decrease in the number of sperm bound to the oolemma (0.3 sperm per egg) when compared to the control group in which oocytes were treated with heat inactivated PI-PLC (21.9 sperm per egg; Fig. 9A). Similarly, PI-PLC treatment also significantly decreased sperm-egg fusion (0.1 sperm per egg) when compared to the control group (2.1 sperm per egg; Fig. 9B). This result indicates that the inhibitory effect of PI-PLC on fertilization is mediated at the oolemma and is in close agreement with results obtained using the mouse in vitro fertilization model (See Examples in Section 6).

Treatment of zona-free eggs with PI-PLC has no effect on artificial egg activation

Artificial activation of oocytes was performed to ensure that the oocytes remained viable following PI-PLC treatment. When eggs were treated with PI-PLC washed and artificially activated with 0.5 μm calcium ionophore A23187, there was no difference ($p < 0.05$) in the percentage of eggs which resumed meiotic cell division when comparing eggs treated with heat inactivated PI-PLC (84%, Fig. 10A) to eggs were treated with active PIPLC (83%, Fig. 10B). The observation that PI-PLC treatment did not alter meiotic

division supports the hypothesis that eggs remained viable following treatment and the PI-PLC effect on sperm-egg binding and fusion was authentic.

A 25-40 kDa (pI 5-6) Protein Cluster is Released from Ocytes treated with PI-PLC

Hamster oocytes were biotinylated and incubated with or without PI-PLC for 30 min. The supernatants were collected from the two groups, the eggs were washed, and the egg proteins were extracted. The egg protein extracts and the proteins from the supernatants were separated by 2-D electrophoresis and electroblotted to nitrocellulose membranes. The membranes were then stained with Protogold to visualize the egg proteins. Next the membranes were probed with strepavidin-HRP and the biotinylated egg surface proteins were visualized using TMB membrane peroxidase substrate. The repertoire of zona-free hamster egg proteins is shown in Fig. 11A with over one hundred egg proteins being resolved following Protogold staining (red staining). The repertoire of surface-labeled zona-free hamster egg proteins (blue staining) can also be seen in Fig. 11A. Approximately eleven biotinylated surface protein spots having molecular weights ranging from ~ 40 to 140 kDa can be visualized (small arrowheads). Seven of these surface labeled protein spots were also stained with Protogold (small arrowheads labeled d). One lesser ~ 45-50 kDa protein cluster c1), one predominant ~25-40 kDa protein cluster c2), and three protein trains having masses of ~ 35, 20 , and 15 kDa (t 1, 2, and 3 respectively) can be resolved. The predominant 25-40 kDa protein cluster c2) can be further resolved into three smaller protein clusters, however, the clusters were not recorded as separate proteins because continuous protein staining was observed between the clusters. The two spots denoted by asterisks in Fig. 10A represent proteins that bound strepavidin-HRP non-specifically and were detected on 2-D blots of oocytes which were not biotinylated.

The repertoire of biotin-labeled egg proteins remaining on the egg surface following PI-PLC treatment is shown in Fig. 11C. The two arrows denote the location of the 25-40 kDa (pI 5-6) protein cluster c2) which is prominent in the extracts of untreated eggs (Fig. 11A) but is absent the extracts of eggs treated with PI-PLC. Note that the staining intensity of the remaining surface labeled egg proteins does not appear to be affected by PI-PLC treatment. In contrast to the supernatant from untreated eggs (Fig. 11B), the supernatant from eggs treated with PI-PLC (arrows, Fig. 11D) reveal a 25-40 kDa (pI 5-6) protein cluster having a similar molecular weight and isoelectric point to that which was released from the egg surface following PI-PLC treatment (Fig. 11C). It is likely that the PI-PLC-sensitive protein cluster seen in Fig. 4D is GPI-anchored and is involved in human sperm-hamster egg binding and fusion. The train of four proteins having a mass of ~29 kDa and indicated by the asterisk in Fig. 11D most probably represents isoforms of PI-PLC

because identical staining patterns are observed when one μg of the PI-PLC preparation is separated on a 2-D gel and silver stained. It is possible that a small amount of unbound biotin remained associated with the biotinylated oocytes following oocyte washing and became linked to PI-PLC during oocyte treatment. This could explain why the PI-PLC isoforms appear to be dually labeled in Fig. 11D.

7.3 DISCUSSION

The findings presented herein demonstrate that PI-PLC has differing effects on human sperm and hamster eggs during gamete interaction. When human sperm were treated with PI-PLC, washed, and incubated untreated zona-free hamster oocytes, sperm-egg binding is significantly enhanced while fusion is not effected. GPI-anchored sperm surface proteins are thought to be involved in processes such as: protection of sperm from the immune response (Kirchhoff and Hale, 1996, Mol. Hum. Reprod. 2: 177-184); the acrosome reaction (Mendoza et al., 1993, J. Cell Biol., 121: 1291-1297); sperm-cumulus interaction (Myles and Primakoff, 1997, Biol. Reprod., 56: 320-327); and sperm-zona pellucida interaction (Mahoney et al., 1991, J. Reprod. Immunol. 19: 269-285; Diekman et al., 1997, Biol. Reprod. 57: 1136-1144). There are no reports in the literature implicating GPI-anchored sperm proteins in sperm-oolemma binding and fusion. In the studies of mouse egg proteins shown in the Example in Section 6, however, we found that when epididymal mouse sperm were treated with PI-PLC, washed, and incubated with untreated zona-free mouse oocytes, there was a slight (but not significant) increase in sperm-egg binding compared to controls, as shown in the Example in Section 6. It is possible that the enhanced increase in sperm-egg binding observed in the present study is due to the fact that ejaculated sperm were treated with PI-PLC as opposed to epididymal sperm which were used in the previous experiment.

One model to explain how treatment of sperm with PI-PLC could cause an increase in sperm-egg binding would posit that PI-PLC treatment releases GPI-anchored proteins from the sperm surface which mask molecules required for sperm-egg binding and fusion. It is known that sperm become coated with GPI-anchored proteins during passage through the epididymis (Kirchhoff and Hale, 1996, Mol. Hum. Reprod. 2: 177-184). It is possible that these proteins act as capacitating factors and are released from the sperm surface during passage through the female reproductive tract. Therefore, in this study, treatment of sperm with PI-PLC may have released more of these GPI-anchored capacitating proteins than were released from the control group, thus leading to enhanced sperm-egg binding. It is also possible that GPI-anchored sperm surface proteins are involved in acrosomal maintenance and loss of these proteins following PI-PLC treatment

increased the percentage of acrosome reacted sperm in the PI-PLC treatment group, thus leading to enhanced binding. Studies are currently underway to establish if PI-PLC affects capacitation or the acrosome reaction.

The most significant finding of this study is that when zona-free hamster oocytes are treated with PI-PLC, washed, and incubated with untreated human sperm, binding and fusion is blocked. While there are no previous reports describing GPI-anchored proteins on mammalian oocytes, there is a GPI-anchored form of N-acetylglucosaminidase which is present of the surface of Ascidian eggs (Lambert, 1989, Development 105: 415-420). This enzyme is PI-PLC-sensitive and is cleaved from the surface of Ascidians eggs following fertilization and occupies sperm binding sites on the vitelline coat to protect the egg against polyspermy (Lambert and Goode, 1992, Dev. Biol. 154: 95-100). Regarding the presence of GPI-anchored proteins on mammalian oocytes, two previous reports have investigated whether treatment of mammalian oocytes with PI-PLC blocks sperm-egg interaction. Clark and Koehier (1988, Gam. Res. 19: 339-348) treated zona-free hamster oocytes with up to 1 U/ml PI-PLC for only 3 min and found that the enzyme had a slight, but significant, inhibitory effect on hamster sperm-hamster egg fusion. However, these results are somewhat difficult to interpret due to the abbreviated treatment time. In our previous study we found that when either zona-intact or zona-free mouse oocytes were treated with PI-PLC, fertilization was blocked. To demonstrate that the effect of PI-PLC was specific to the release of GPI-anchored proteins, we also performed several control experiments. As with the hamster oocytes in this study, PI-PLC treated mouse oocytes are fully capable of being artificially activated, thus indicating that the oocytes are viable following treatment. Also, the decrease in mouse sperm-egg binding and fusion depended on the dose of PI-PLC employed, with a maximal inhibitory effect on binding and fusion at 1 U per ml. Finally, treatment of oocytes with PI-PLC did not reduce the immunoreactivity of the non-GPI-anchored egg surface integrin, $\alpha 6\beta 1$, as shown in the Example in Section 6. Therefore, in vitro data from our previous study as well as this study indicate that there is a PI-PLC-sensitive GPI-anchored protein(s) on the mammalian oocyte which is required for sperm-egg binding and fusion.

In this study, the repertoire of biotinylated hamster oolemmal proteins has been resolved using 2-D gel electrophoresis followed by avidin blotting. Results show that approximately eleven isolated protein spots, two protein clusters, and three protein trains are surface labeled (Fig. 11A). The two protein clusters (c1 and c2) represent separate proteins each with multiple isoforms containing varying degrees of glycosylation while the protein trains (t1, t2, and t3) represent non-glycosylated proteins each consisting of multiple isoforms (Shackelford et al., 1980, J. Exp. Med. 151: 144-165; Negm et al., 1991, Comp.

Biochem. Phys. Comp. Biochem. 99: 741-749). The number of surface labeled proteins which can be visualized on the 2-D avidin blots of hamster oocytes is consistent with results obtained from one dimensional blots of surface labeled mouse (Boldt et al., 1989, Gam. Res. 23: 91-101; Flaherty and Swan, 1993, Mol. Reprod. Dev. 35: 285-292; Ya Zhong et al., 1997, Mol. Reprod. Dev. 47: 120-126) and hamster (Ya Zhong et al., 1997, Mol. Reprod. Dev. 47: 120-126) oocytes. Also, although the 2-D repertoire of hamster oolemmal proteins is quite similar to that which was observed in the mouse, shown in the Example in Section 6, herein, there were notable differences in the masses of the protein clusters. In the mouse, the predominant oolemmal protein cluster is ~ 70 kDa with a less prominent protein cluster seen at 35-45 kDa. The hamster oolemma, on the other hand, contains a predominant protein cluster at ~25-40 kDa with a less prominent protein cluster at 45-50 kDa.

A most striking observation is that the predominant 25-40 kDa (pI 5-6) protein cluster (c2) which is evident in the 2-D avidin blots of untreated oocytes (Fig. 11A) is absent from the blot in which hamster oocytes were treated with PI-PLC (Fig. 11B). Further, the supernatant from eggs treated with PI-PLC (arrows, Fig. 11D) revealed a protein cluster of similar molecular weight and isoelectric point (25-40 kDa, pI 5-6) to that which was released from the eggs surface following PI-PLC treatment (Fig. 11D). The enzyme appears to be specifically affecting the 25-40 kDa protein cluster (c2) because the staining intensity of the remaining surface labeled proteins in Fig. 11C is similar to that which is seen in the untreated eggs in Fig. 11A. Of interest is the observation that, in the hamster, the predominant protein cluster (c2) is PI-PLC sensitive while the less prominent protein cluster (c1) is not affected by PI-PLC treatment (see Fig. 11B). However, in the mouse, both the 70 kDa and 35-40 kDa protein clusters are PI-PLC sensitive (see Example in Section 6).

In conclusion, when human sperm are treated with PI-PLC, sperm-egg binding is enhanced while sperm-egg fusion is not effected. When zona-free hamster oocytes are treated with PI-PLC, sperm-egg binding and fusion is blocked. Results from the 2-D avidin blots show that a predominant protein cluster is released from the hamster oolemma following PI-PLC treatment. This PI-PLC-sensitive protein cluster (~25-40 kDa, pI 5-6) is thus likely to mediate human sperm-hamster egg binding and fusion.

8. EXAMPLE: The Identification of a Novel Membrane-Bound Form of ZP-3

The experiments presented in the Example herein demonstrate the successful identification of a membrane-bound form of the zona pellucida sperm-binding protein, ZP3. ZP3 is a well known highly conserved, zona pellucida protein which has been characterized in many species and whose nucleotide and protein-coding sequences are known (for e.g., mouse GenBank Accession No. M20026; rat GenBank Accession No. Y10823; zebrafish AA566910; pig L22169; PCT patent no. WO9410304). The mRNA (SEQ ID NO:1) and protein (SEQ ID NO:2) sequence of human ZP3 is shown in Fig. 12. ZP3 is responsible for primary binding of the egg to a receptor(s) on the sperm (reviewed in McLeskey et al., 1998, Int. Rev. of Cytol. 177: 57-113). This binding event also initiates the acrosome reaction in which hydrolytic enzymes are released from the acrosomal compartment and act on the zona pellucida to facilitate penetration of the zona pellucida by sperm.

Because the GPI-linked M70 egg surface protein isolated from mouse eggs is similar to ZP-3 in size, the following experiment was carried out to determine whether M70 is a membrane-associated GPI-linked form of ZP3. Either zona-free mouse oocytes or mouse zona pellucidae were extracted in Triton-X114 and the detergent and aqueous phases were isolated. The samples were subjected to SDS-PAGE and Western blot analysis using the monoclonal antibody (mAb) (IE-10). As shown in Figure 13A, the oolemmal form of ZP3 partitions in the detergent phase. This indicates that oolemmal ZP3 is associated with the membrane and contains a membrane-associated domain. On the other hand, as shown in Fig.13B, zona matrix AP3 partitions in the aqueous phase, indicating that this form of ZP3 is soluble. These results further show that the mass of oolemmal ZP3 is less than that of matrix ZP3.

In Fig. 14, localization of SP3 to the oolemma of zona-free mouse oocytes was demonstrated by immunostaining with ZP3 mAb . The zonae pellucida were removed from ovulated mouse oocytes by treatment with chymotrypsin followed by mechanical agitation. As a control, the eggs were first treated with 10μg/ml Hoechst dye, which stains chromatin in blue. The eggs were then incubated with either 20μg/ml rat IgG or purified anti-ZP3 mAb (IE-10), washed 3 times, and incubated with fluorescent (CY-3)-conjugated goat anti-rat secondary antibodies. As shown in Fig. 14, oolemmal ZP3, which is stained in red, is localized to the oocytes microvillar region of oocytes. Similar results were seen when the zonae were removed with either acid Tyrodes or mechanical shearing.

Next, in order to characterize the membrane-bound form of ZP-3, kidney cells were transfected with full length recombinant mouse ZP3 (Genbank Accession No. M20026). Mouse ZP3 was cloned into a mammalian expression vector by cleaving pZP3.4 (Ringuette et al., Dev. Biol. 127: 287-295) with HindIII and EcoRI and ligating the ZP3-

encoding fragment into a pcDNA3.1 expression vector. Kidney cells were transiently transfected with the resultant construct using Transit (Panvera, Madison, Wisconsin). The cells were fixed with paraformaldehyde, incubated with the anti-ZP3 mAb, IE-10, and a fluorescently labeled secondary antibody. As shown in Fig.15, punctate cell surface staining pattern can be seen on cells which expressed the cDNA, while cells not expressing ZP3 do not react with the IE-10 mAb. At higher magnification, as shown in the center panel, distinct types of punctate staining can be visualized. The absence of staining on cells which do not express ZP3, indicates that ZP3 is specifically associated with the plasma membrane and is not adventitiously binding to the cell surface following secretion into the culture medium.

In a final demonstration of the membrane-associated ZP3, a peptide (J peptide) corresponding to the IE-10 mAb epitope on ZP3 was synthesized. The IE-10 mAb was incubated with either no peptide or saturating concentrations of the peptide prior to incubation with zona-free mouse oocytes. The eggs were then washed and incubated with fluorescently tagged secondary antibody. Indirect immunofluorescence images show that the peptide blocked antibody-antigen recognition, indicating that the IE-10 mAb was specifically recognizing ZP3 on the oolemmal surface.

In conclusion, the experiments described in this Example demonstrate the existence of a membrane-anchored form of ZP3. These results, taken together with the release of a 70 kDa protein upon treatment of mouse oocytes with PI-PLC, suggests the existence of a GPI-anchored form of ZP3. This GPI-linked, membrane bound, ZP3 is a potentially powerful antigen that could be used as a contraceptive vaccine. In particular, a GPI-linked human ZP-3 antigen, comprising a GPI-linked ZP3, or fragment thereof, would be useful as a human contraceptive.

9. EXAMPLE: Identification of a Subset of Egg Proteins Which Localize to the Egg Surface

The following Example demonstrates the identification and characterization of egg surface proteins. In particular, a novel sequence of a novel egg surface protein has been identified. These sequences can be used as antigenic molecules for the compositions and methods of the present invention.

Two-dimensional gel electrophoretic analysis of one thousand zona-free mouse oocytes was performed to determine the extent to which oolemmal proteins could be resolved on silver stained 2-D gels. In this experiment, zona-pellucidae were removed from the ovulated mouse oocytes using acid Tyrodes and the egg proteins were solubilized in a

non-ionic detergent/urea lysis buffer. The proteins were separated by isoelectric focusing followed by polyacrylamide gel electrophoresis. The gel was then silver stained to visualize egg proteins. Over five hundred oocyte proteins can be visualized on this gel. The approximate isoelectric points (pI) of the proteins and the approximate molecular masses (kDa) of the proteins have been determined. Most of the egg proteins fall within a pI range of 5-6.

Two-dimensional gel electrophoresis followed by avidin blotting was utilized to determine if vectorially labeled mouse oolemmal proteins could be resolved and visualized. Eight hundred zona-free mouse oocytes were surface labeled with Sulfo-NHS biotin and extracted with a non-ionic detergent. The extracted egg proteins were separated by 2-D electrophoresis, electroblotted to nitrocellulose membranes, probed with streptavidin-HRP, and biotinylated proteins were visualized on radiograms using enhanced chemi-luminescence. The 2-D gel repertoire of biotin-labeled egg surface proteins is presented in Fig. 1B. At least fifty isolated protein spots can be visualized with most falling in the 35-100 kDA (pI 5-6) region of the blot. Of particular interest are the three large protein clusters centered at ~70 kDa (pI 5), 40 kDa (pI 5.5) and 27 kDa (pI 5). It is likely that these clusters represent isoforms of heavily glycosylated proteins. To our knowledge, this is the first report of surface-labeled oocyte proteins being resolved on 2-D blots.

To ensure that plasma membrane proteins were biotinylated and cytoplasmic proteins were not, the blot described above was stripped of streptavidin-HRP and re-probed with antibodies to tubulin and actin. No surface-labeling of these cytoskeletal proteins occurred, validating the surface-specificity of the labeling method. Tubulin antibody localized precisely to a spot which, while surrounded by avidin-staining, is devoid of any labeling. To ensure that strepavidin was specifically recognizing Sulfo-NHS biotinylated egg proteins, 2-D blots of non-biotinylated oocytes were probed with strepavidin-HRP. Two proteins in the unlabeled eggs bound streptavidin.

9.1 Microsequencing Surface-labeled Proteins

With the preliminary oocyte proteomic database in place, and a subset of egg surface labeled proteins identified, microsequence information was obtained from several of the surface-labeled proteins. For the microsequencing experiments, one hundred zona-free mouse oocytes were biotinylated, separated on 2-D minigels, and the surface-labeled proteins were visualized by avidin blotting. Concurrent with the surface-labeling experiment, similar numbers of oocytes were separated on 2-D minigels and silver-stained. Coordinates of several surface-labeled proteins directly matched those of silver-stained proteins indicating that these oocyte proteins localized to the cell surface. It is known that

some glycoproteins cannot be visualized by standard silver staining techniques. The heterogeneous nature of the 70 and 35-45 kDa protein clusters (double arrowhead pointing to the right) suggest that these proteins may be heavily glycosylated which may account for their lack of silver staining. Four putatively surface labeled proteins were chosen for microsequencing based on their consistent labeling with biotin after multiple surface-labeling experiments.

Microsequence data was obtained using LC-MS tandem mass spectrometry (Wilm *et al.*. 1996). To ensure that sufficient protein concentrations would be available for this sequencing experiment, proteins from approximately 1300 zona-free oocytes were separated on a 2-D minigel and the gel was Coomassie-stained. The selected surface-labeled proteins spots were then cored from the minigel and submitted to the Mass Spectrometry Laboratory for microsequence analysis.

The resulting microsequence information is summarized below:

TABLE 1

Protein Identification	Protein Coordinates	% of Known Sequence	Type of Membrane Attachment	First Time Identified in Mammalian Egg
1 = Novel	MW 120, pI 4.3	N/A	N/A	N/A
2 = Calnexin	MW 107, pI 4.4	6%	integral	yes
3 = HSP 78 (BIP)	MW 78, pI 4.82	30%	none reported	yes
4 = Calreticulin	MW 64, pI 4.2	37%	associated with KDEL docking protein	yes

Protein # 1 - Three peptide sequences were deduced from the CAD spectra of ions from the tryptic digest of Protein #1. Database searches using Sequest could not identify these peptides in any database sequence. To validate the authenticity of these peptide sequences, protein # 1 was cored from another Coomassie stained gel containing similar numbers of extracted oocytes and re-submitted to the Mass Spectrometry Lab for microsequencing and identical results were obtained. Therefore, protein # 1 is novel and a prime candidate for cloning and characterization.

TABLE 2

Peptide Sequences From Protein #1 (NOVEL)

Peptide No.	Measured Molecular Weight ($M+H^+$, Da)		Peptide Sequence by CAD[1] (calculated MW, $M+H^+$, Da)	Peptide Sequence from Database (calculated MW, ($M+H^+$, Da)
1	843.6	+2		SFSDFLK (843.4)
2	1023.8	+2	uninterpretable	
3	1364.0	+2	__XPEATG ---K $\overline{b2}$ = 169	
4	1369.6	+2	__SXVNVS---	

[1] X designates I or L which cannot be distinguished by low energy CAD,
M(o) designates oxidized M,
C* designates carbamidomethyl modified C,
_ indicates an unknown residue,
- - - indicates an unknown number of unknown residues.

Proteins # 2, 3, and 4 - Sequence obtained from protein spots 2. 3, and 4 corresponded to calnexin, HSP78 (BIP), and calreticulin respectively. These proteins fall into a class of proteins which bind calcium and function as molecular chaperones. Molecular chaperones modulate the folding and assembly of newly synthesized proteins and protein complexes (Wynn *et al.*, 1994, J. Lab Clin. Med., 124:31-36). Calnexin and its soluble homologue, calreticulin, specifically modulate glycoproteins (Oliver *et al.*, 1996, J. Biol. Chem. 271:13691-96). Many cell surface proteins are glycoproteins and exist as complexes of multiple subunits. These subunits are thought to be assembled by molecular chaperones in the endoplasmic reticulum (Van Leeuwen and Kearse, 1996, J. Biol. Chem. 271:25345-49). There is a growing amount of evidence that, in some cases, the chaperone proteins are not released from the plasma membrane complexes with which they are assembling and are carried to the cell surface where they may be engaged in alternate functions than those which they perform in the ER. As an example, BIP, calnexin, and calreticulin assist in assembly of the T-cell antigen receptor complex (TCR) in the ER (Van Leeuwen and Kearse, 1996, *supra*). In thymocytes, calnexin maintains its association with CD3 (a component of the TCR) and the complex is expressed on the cell membrane of immature thymocytes where it is thought to play a role in cell signaling (Wiest *et al.*, 1995, EMBO J. 14:3424-33).

The highly conserved heat shock protein (HSP) family has generally been thought to function intracellularly. However, recent evidence suggest that, in some instances, these proteins are also expressed on the plasma membrane. For example, in some

types of T-cells, HSPs, are thought to actually function as antigen presenting molecules (Multhoff *et al.*, 1995, Int. J. Cancer, 10:272-9). They are also present on the surface of T-cells and mononuclear cells which are undergoing apoptosis (Poccia *et al.*, 1996, Immunology, 88:6-12). HSPs have also been implicated in fertilization (Boulanger *et al.*, 1995a, J. Cell Physiol. 165:7-17). The gene encoding the egg plasma membrane receptor for sperm was cloned in the sea urchin and the extracellular domain of the receptor is similar to the HSP70 family of proteins (Foltz *et al.*, 1993, Zygote, 1:276-9). Heat shock proteins have also been found on the surface of sperm by our lab and others (Boulanger *et al.*, 1995b, *supra*). Regarding BIP specifically, one function of this molecule is known to bind to and assist in the finding that one of the surface-localized oocyte proteins was calreticulin was of particular interest. Following the acrosome reaction, calreticulin is expressed on the plasma membrane overlying the equatorial segment of sperm. Because sperm-egg fusion is thought to initiate in this region of sperm, it is possible that sperm surface calreticulin is involved in this aspect of gamete interaction.

Recently, the cell surface protein, Clq-R, was found to have almost complete amino acid sequence identity with calreticulin (Stuart *et al.*, 1997). Clq-R is a receptor for Clq, a component of the classical complement pathway and has been found on many cell types (Peterson *et al.*, 1997) including oocytes(Fusi *et al.*, 1991) and sperm (Bronson *et al.*, 1998). One known function of cell-surface calreticulin is to mediate adhesion to extracellular matrix proteins such as laminin and fibrinogen (Gray *et al.*, 1995; McDonnell *et al.*, 1996; White *et al.*, 1995). This adhesion process is thought to occur in association with integrins such as $\alpha 6\beta 1$ (Zhu *et al.*, 1997). Given calreticulin's implicated role in fertilization, surface localization on sperm following the acrosome reaction, association with integrins, and confirmed cell surface expression in other cell types, we decided to use calreticulin as a model protein and proceed to the next aim of this grant proposal for known proteins, which is to investigate the role of identified oolemmal proteins in fertilization using in vitro fertilization assays.

The specificity of the rabbit polyclonal antibody to full length recombinant calreticulin used for the subsequent assays was confirmed by probing a 2-D blot of zona-free mouse oocytes. The blot was first stained with Protogold to visualize oocyte proteins (Fig. 3A). Immunoblotting revealed that the calreticulin antibodies are highly specific and recognize only two oocyte proteins, calreticulin and calnexin (Fig. 3B, arrows). It is not surprising that the calreticulin polyclonal antibody also recognized calnexin because calnexin is the membrane bound homologue of calreticulin. However, because both proteins were recognized by the calreticulin antibody, it is understood that the results presented below could be due to antibody reactivity with either calreticulin, calnexin, or

both. Fig. 3C highlights the observation that both calreticulin and calnexin were surface-labeled with biotin. The oocyte cell surface localization of calreticulin was then confirmed by indirect immunofluorescence. A greater fluorescence staining intensity was observed on live zona-free oocytes that were incubated with fluorescent beads coated with a 1-50 dilution of calreticulin antisera when compared to oocytes incubated with beads coated with a similar dilution of normal rabbit sera.

The effect of calreticulin antibodies on sperm-egg interaction was then investigated in an in vitro fertilization assay. Cumulus-free zona-intact mouse oocytes were either not treated with antibodies or treated with a 1:50 dilution of normal rabbit sera or anti-calreticulin antibodies. The eggs were then washed and inseminated with untreated mouse epididymal sperm. Following overnight incubation, oocytes which had not undergone cleavage were scored as unfertilized and oocytes which bad cleaved were scored as fertilized. Significantly fewer ($p < 0.05$) oocytes underwent cleavage following incubation with calreticulin antibodies (1.3%) when compared to oocytes which were either not treated (68%) or treated with normal rabbit sera (75%).

It appears that the effect of calreticulin antibodies on fertilization occurs following sperm-egg fusion and prior to syngamy. Interestingly, calreticulin has been shown to modulate calcium transients following engagement of surface integrins (Coppolino *et al.*, 1997) and when calcium transients are blocked, fertilization becomes arrested following sperm-egg fusion in both sea urchins (Lee and Shen, 1998) and mice (Xu et al., 1994).

The invention is not to be limited in scope by the specific embodiments described which are intended as single illustrations of individual aspects of the invention, and functionally equivalent methods and components are within the scope of the invention. Indeed various modifications of the invention, in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

All references cited herein are incorporated by reference herein in their entireties for all purposes.

WHAT IS CLAIMED IS:

1. An antigenic molecule comprising an isolated egg surface protein covalently linked to glycosylated phosphatidylinositol moiety and having sperm binding activity.

2. An antigenic molecule of Claim 1, wherein the isolated egg surface protein has the following characteristics: 1) a molecular weight of 70kDa; 2) a pI of 5; and 3) the ability to be specifically released from the egg surface upon treatment with phosphatidylinositol-specific phospholipase C.

3. An antigenic molecule of Claim 1, wherein the isolated egg surface protein has the following characteristics: 1) a molecular weight of 1) a molecular weight between 35 and 45 kDa ; 2) a pI of 5.5; and 3) the ability to be specifically released from the egg surface upon treatment with phosphatidylinositol-specific phospholipase C.

4. An antigenic molecule of Claim 1, wherein the isolated egg surface protein has the following characteristics: 1) a molecular weight between 25 and 40 kDa; 2) a pI between 5 and 6; and 3) the ability to be specifically released from the egg surface upon treatment with phosphatidylinositol-specific phospholipase C.

5. An antigenic molecule of Claim 1 wherein the isolated egg surface protein comprises ZP3, or a fragment or analog thereof covalently linked to a glycosyl-phosphotidyinositol moiety.

6. An antigenic molecule of Claim 1 wherein the isolated egg surface protein comprises the amino acid sequence of SEQ ID NO:2, or a fragment or analog thereof covalently linked to a GPI moiety.

7. A method for preparing antiserum comprising an antibody to an egg surface protein, said method comprising:

(a) immunizing an animal with an egg surface protein or an immunogenic fragment thereof;

(b) obtaining serum from the immunized animal;

(c) screening the serum for the ability to bind to an egg surface protein or an antigenic fragment thereof; and

(e) recovering serum with said ability.

8. A purified antiserum produced by the method of Claim 7 which comprises a polyclonal antibody.

9. An antiserum specific to the antigenic molecule of Claim 1.

10. A method for preparing a monoclonal antibody to an egg surface protein comprising:

(a) immunizing an animal with an egg surface protein;

(b) obtaining antibody-secreting cells from the immunized animal;

(c) immortalizing the antibody-secreting cells obtained in step (b) to produce immortalized cells producing monoclonal antibodies;

(d) screening the immortalized cells for the ability of their secreted antibodies to bind to the egg surface protein, or to inhibit *in vitro* fusion and fertilization, or to bind to an egg surface protein or an antigenic fragment thereof; and

(e) recovering the antibody secreted by the immortalized cells with said ability.

11. A purified antibody produced by the method of Claim 6.

12. An isolated antibody specific to the antigenic molecule of Claim 1.

13. A method for screening a molecule for activity to modulate the level of egg surface protein activity comprising contacting cells with the molecule, and comparing the level of egg surface protein, mRNA or activity in cells contacted with the molecule to the amount of egg surface protein, mRNA, or activity, in cells not so contacted, wherein an increase or decrease in the amount of egg surface protein, mRNA, or activity in the contacted cells relative to the amount of egg surface protein, mRNA, or activity in the cells not so contacted indicates that the molecule has activity to modulate egg surface levels or activity.

14. A method for screening a molecule for the ability to interact with an egg surface protein comprising contacting the egg surface protein with one or more molecules under

conditions conducive to complex formation between the egg surface protein and the molecule, and recovering a molecule that binds specifically to the egg surface protein.

15. A method for modulating fertility comprising administering to a subject a therapeutically effective amount of any of the antigenic molecules of Claims 1-6.

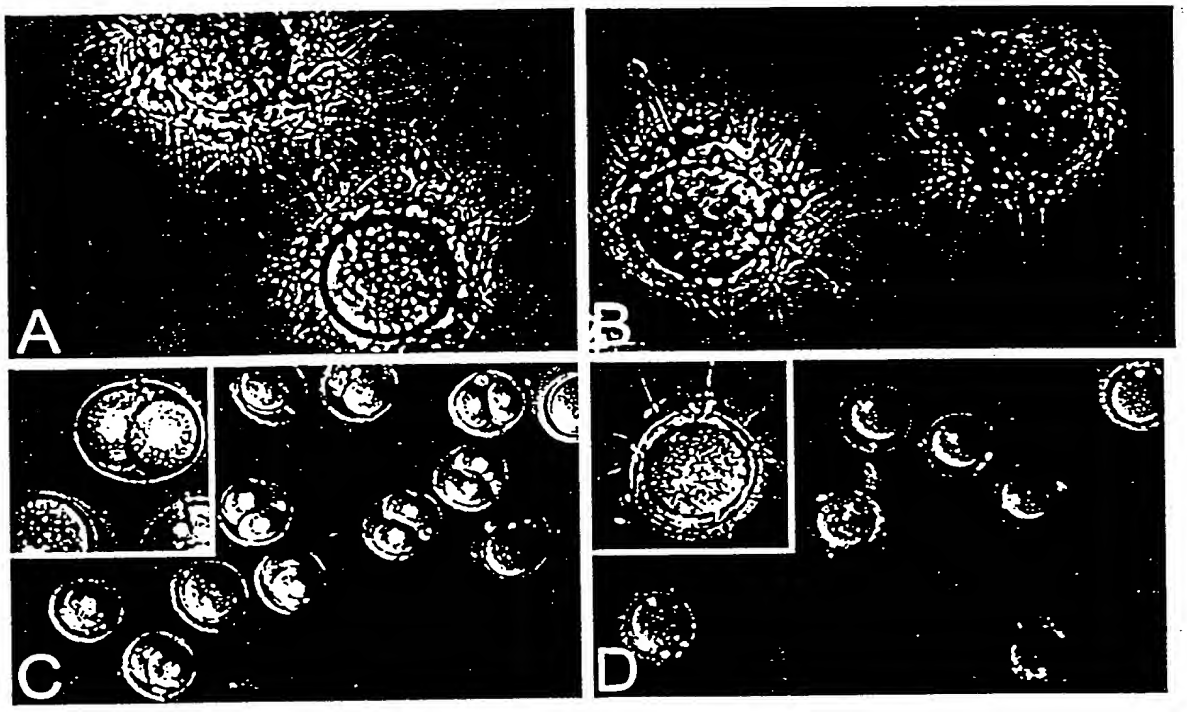
16. A method for modulating fertility comprising administering to a subject a therapeutically effective amount of an antiserum of Claims 8-9.

17. A method for modulating fertility comprising administering to a subject a therapeutically effective amount of an antibody of Claim 11-12.

18. A method for modulating fertility comoprising administering to a subject a therapeutically effective amount of a molecule that modulates the activity of an egg surface protein.

Abstract

The present invention is directed to egg surface antigens useful for producing antibodies which bind epitopes on the egg surface and modulate fertility. The invention encompasses compositions and methods for immunizing an individual for production of antibodies against egg surface antigens. The invention is based on the discovery of egg surface antigens involved in egg-sperm binding and fusion. Methods are provided for the use of such antigens in methods for sterilization of female animals. Methods are further provided for the use of egg surface antigens to generate antibodies useful for temporary, reversible contraception methods. Methods are further provided for the use of anti-idiotypic monoclonal antibodies which mimic egg surface epitopes to actively immunize a mammal against pregnancy.



FIGS. 1A - 1D

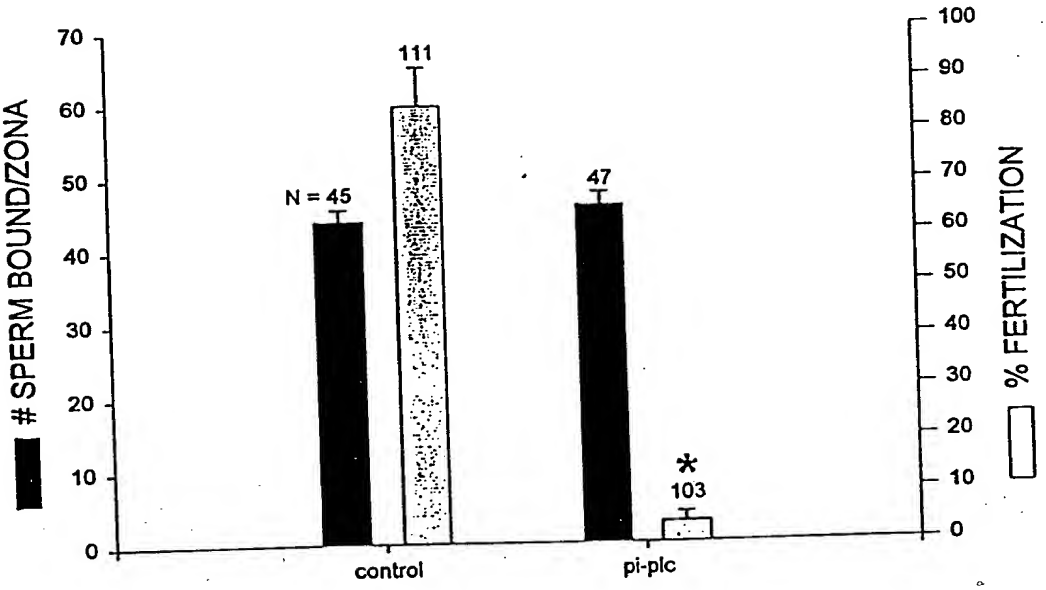
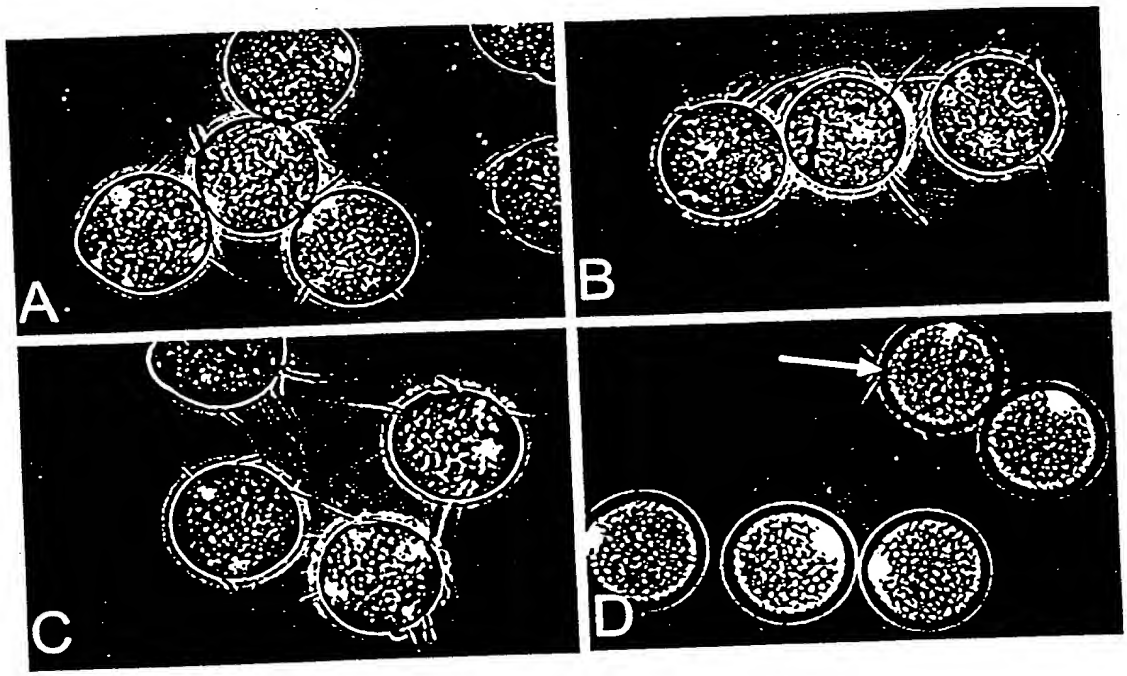

SPERM BOUND/ZONA
% FERTILIZATION
70
60
50
40
30
20
10
0
100
90
80
70
60
50
40
30
20
10
0
N = 45
111
47
*
103
control
pi-plc

FIG. 1E



FIGS. 2A-2D

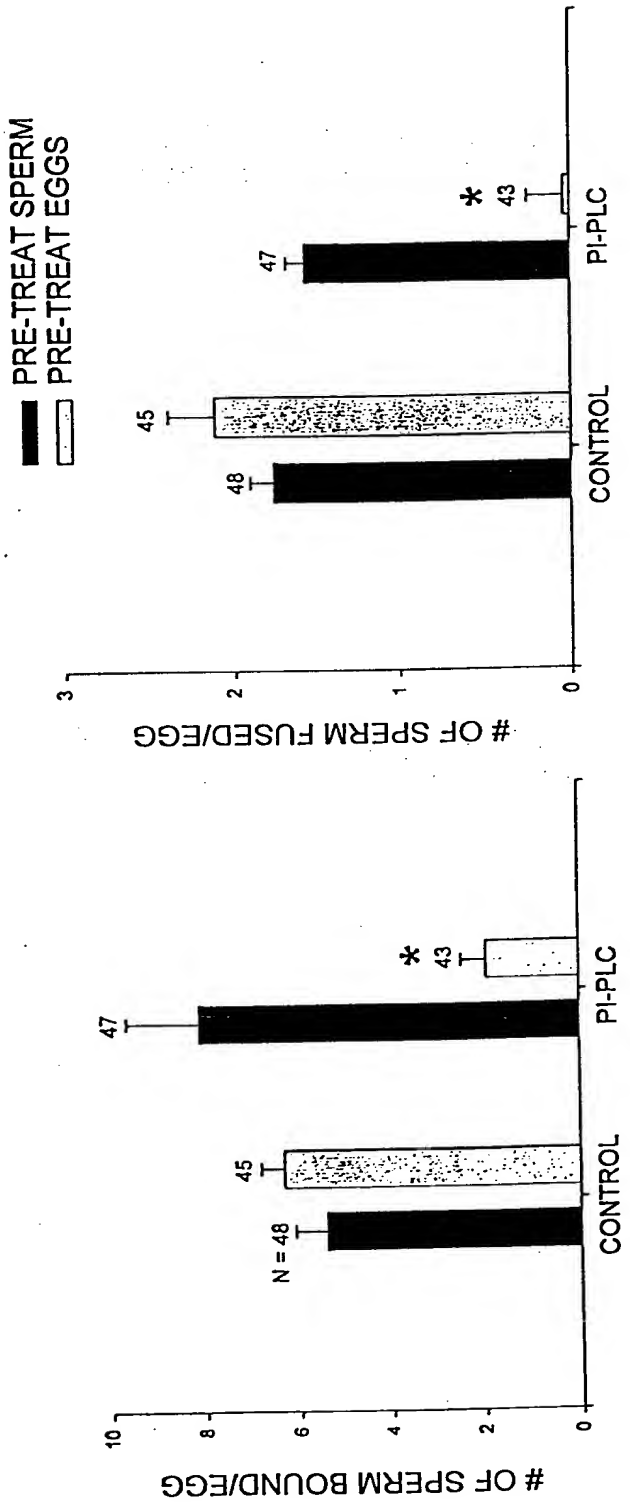

PRE-TREAT SPERM
PRE-TREAT EGGS
OF SPERM FUSED/EGG
0
1
2
3
48
45
47
43
*
CONTROL
PI-PLC
OF SPERM BOUND/EGG
0
2
4
6
8
10
N = 48
45
47
43
*
CONTROL
PI-PLC

FIG. 2E

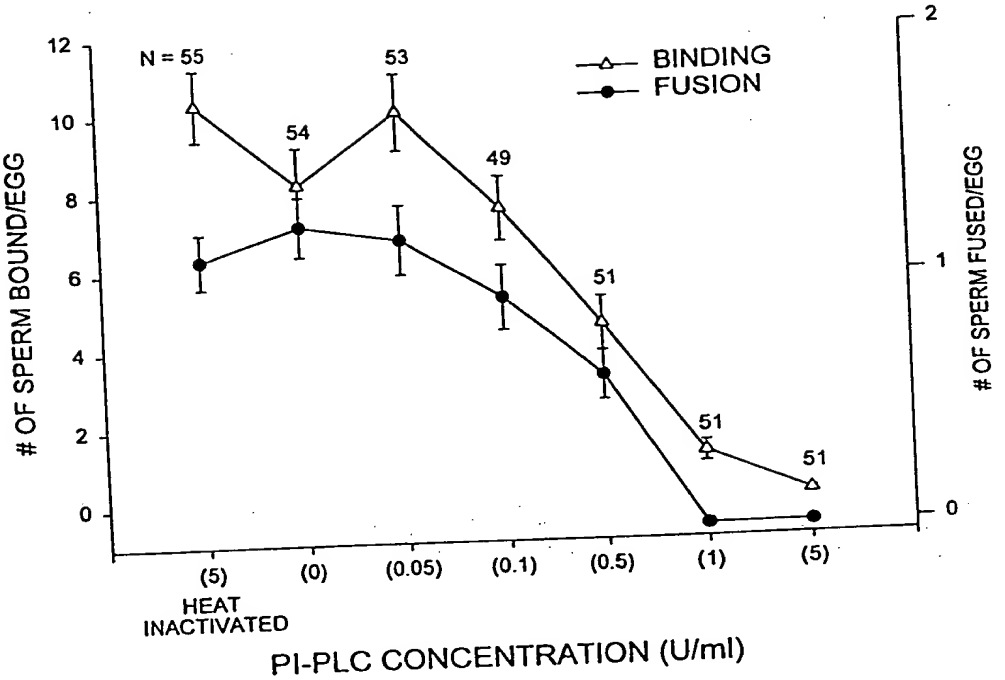

N = 55
53
54
49
51
51
51
BINDING
FUSION
OF SPERM BOUND/EGG
OF SPERM FUSED/EGG
12
10
8
6
4
2
0
2
1
0
(5)
HEAT INACTIVATED
(0)
(0.05)
(0.1)
(0.5)
(1)
(5)
PI-PLC CONCENTRATION (U/ml)

FIG. 3

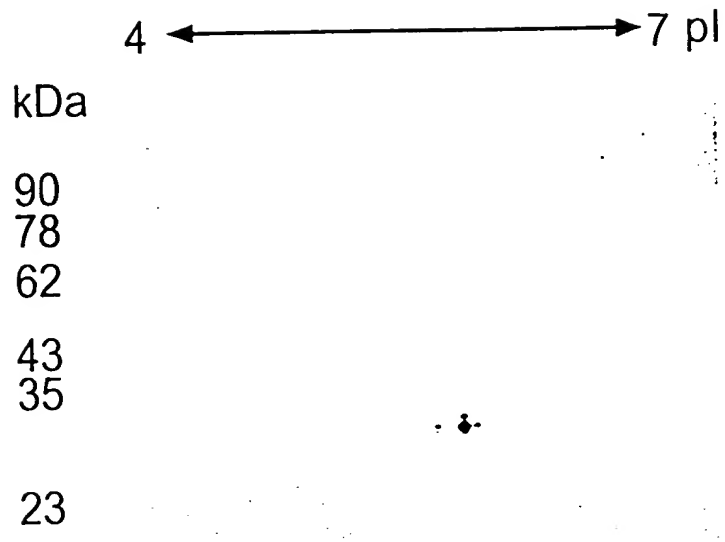


FIG. 4

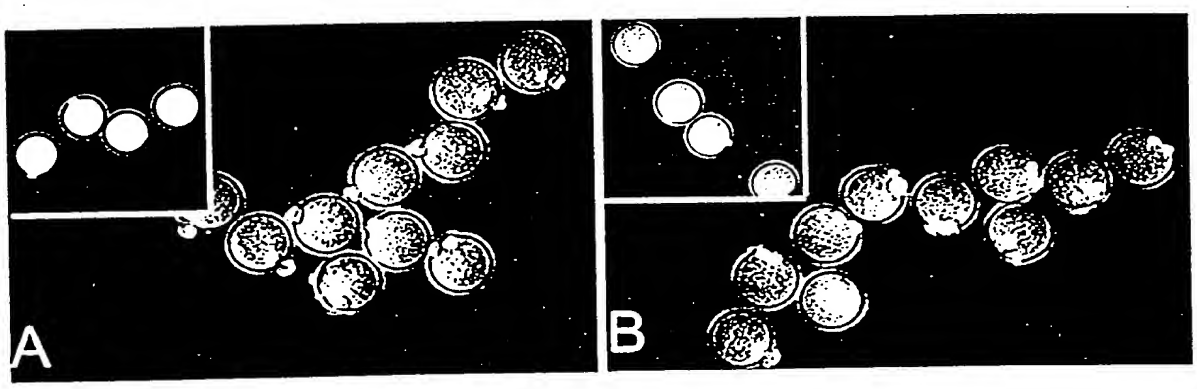


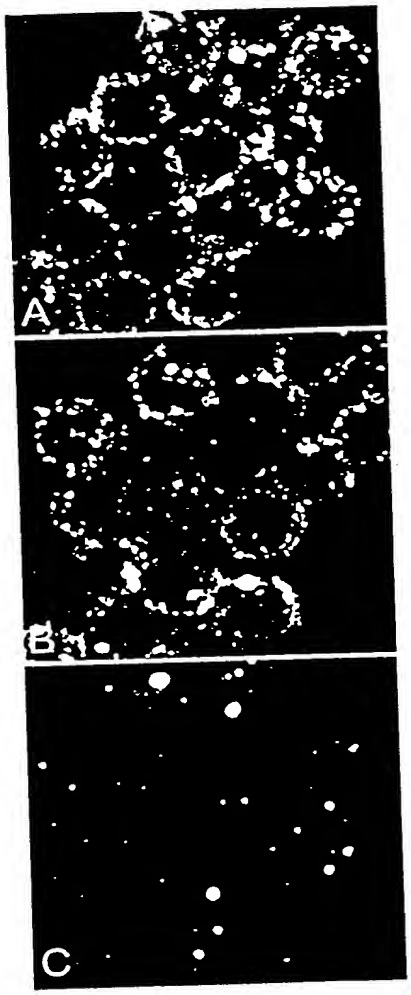
FIG. 5A

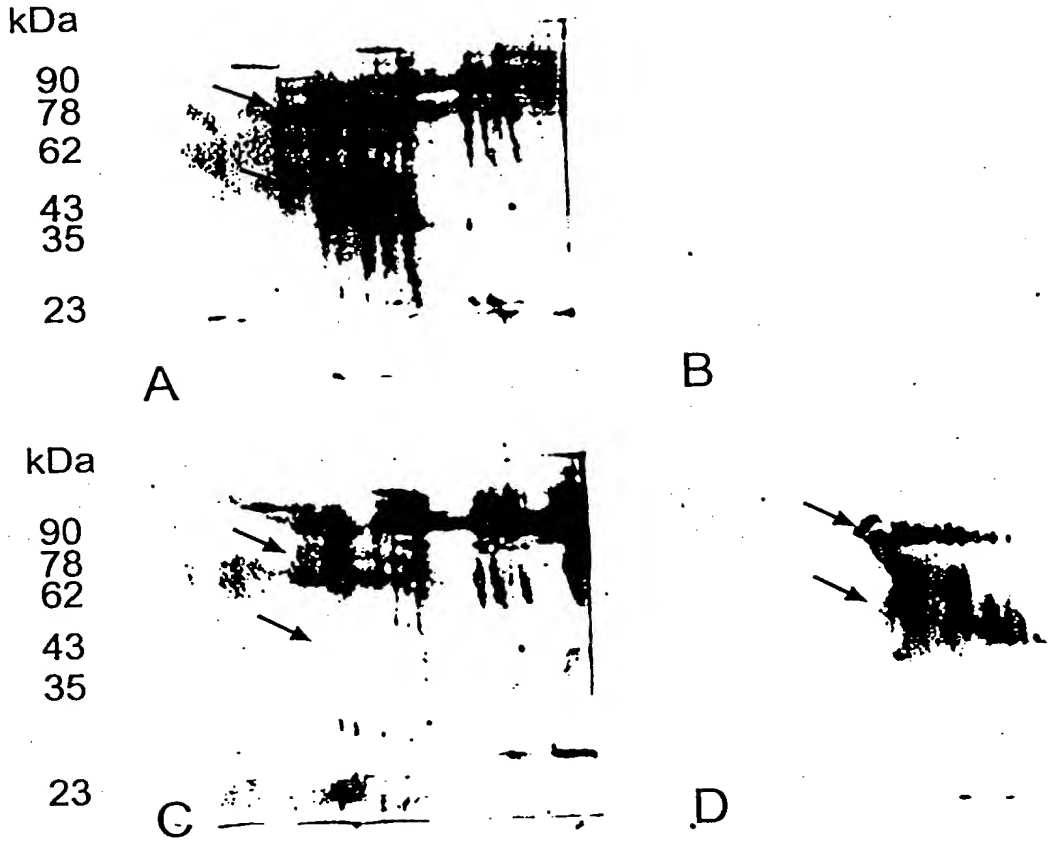
FIG. 5B

FIG. 6A

FIG. 6B

FIG. 6C





FIGS. 7A - 7D

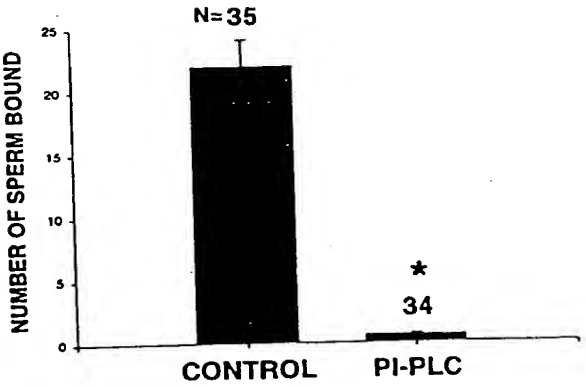

N=35
NUMBER OF SPERM BOUND
25
20
15
10
5
0
*
34
CONTROL
PI-PLC

FIG. 8A

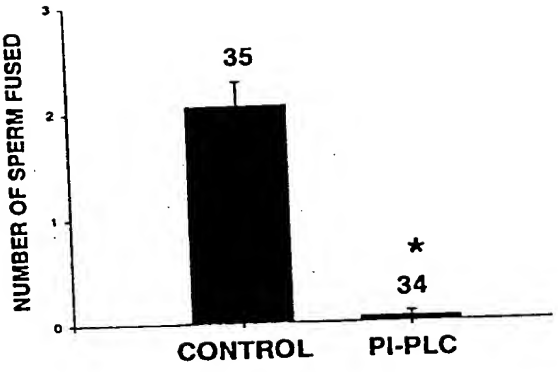

NUMBER OF SPERM FUSED
3
2
1
0
35
*
34
CONTROL
PI-PLC

FIG. 8B

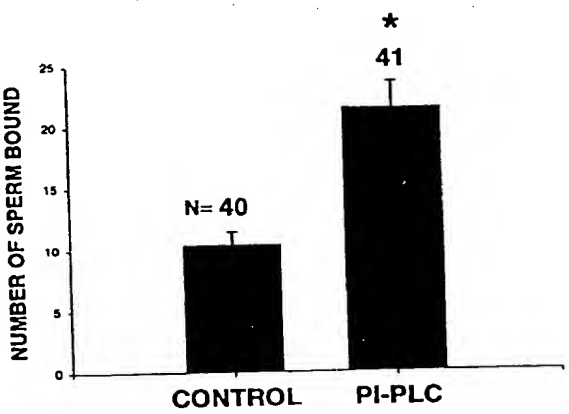

*
41
NUMBER OF SPERM BOUND
25
20
15
10
5
0
N= 40
CONTROL
PI-PLC

FIG. 9A

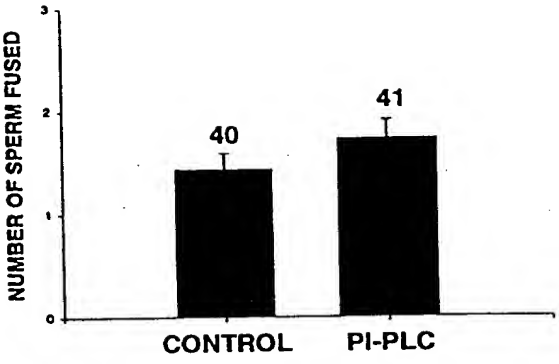

NUMBER OF SPERM FUSED
3
2
1
0
40
41
CONTROL
PI-PLC

FIG. 9B

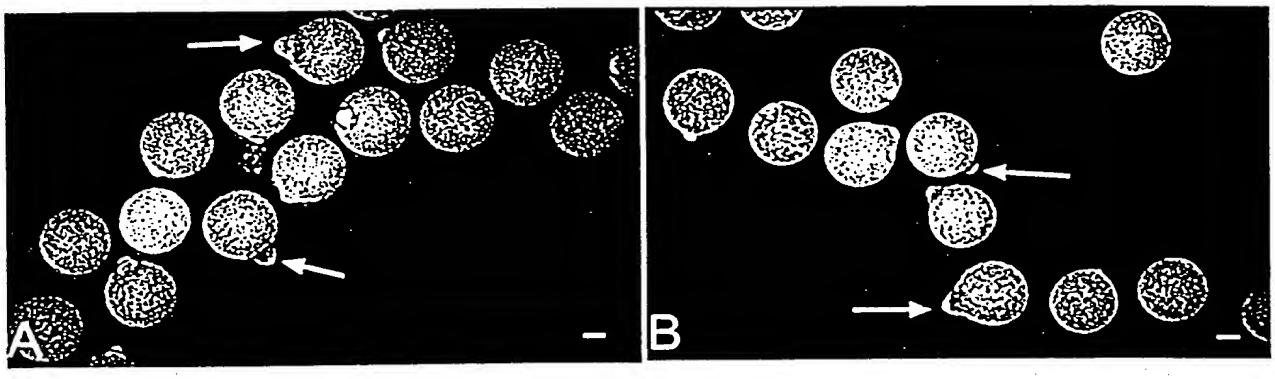
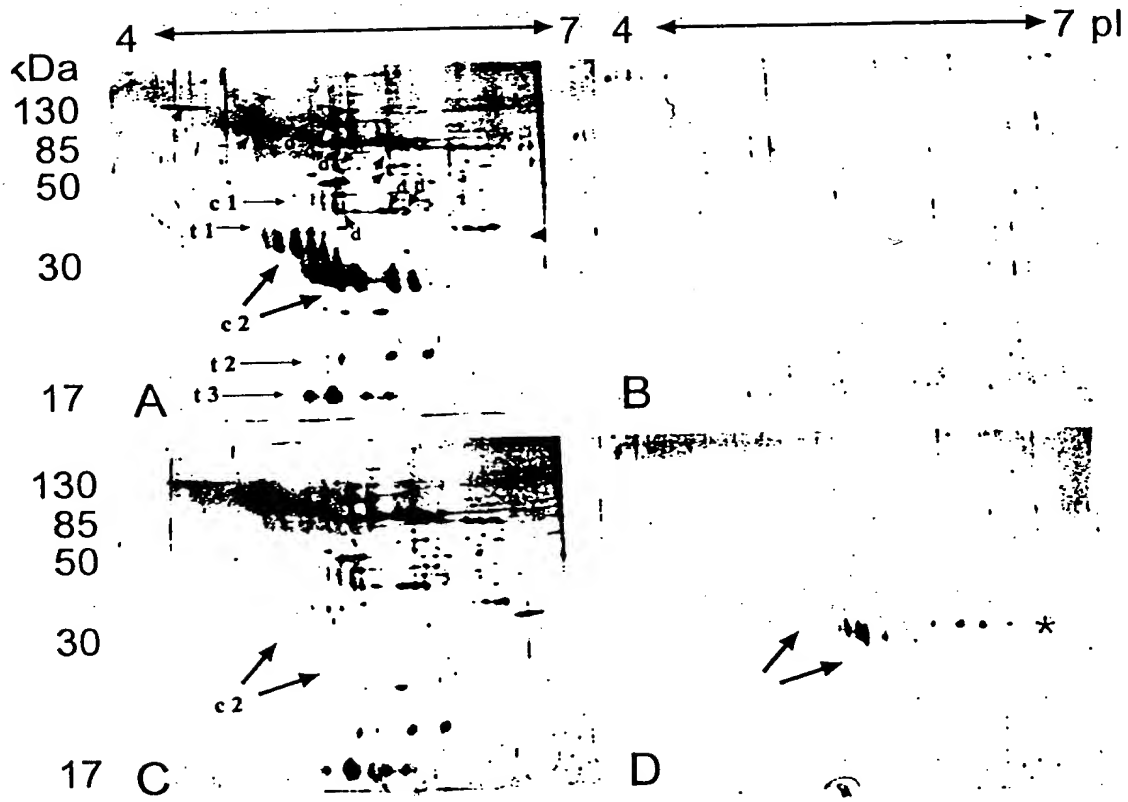


FIG. 10A

FIG. 10B



FIGS. 11A-11D

```
   1 gaggcggctg cctgctgctc tgcaggtacc atggagctga gctataggct cttcatctgc
  61 ctcctgctct ggggtagtac tgagctgtgc tacccccaac ccctctggct cttgcagggt
 121 ggagccagcc atcctgagac gtccgtacag cccgtactgg tggagtgtca ggaggccact
 181 ctgatggtca tggtcagcaa agacctttt ggcaccggga agctcatcag ggctgctgac
 241 ctcaccttgg gcccagaggc ctgtgagcct ctggtctcca tggacacaga agatgtggtc
 301 aggtttgagg ttggactcca cgagtgtggc aacagcatgc aggtaactga cgatgccctg
 361 gtgtacagca ccttcctgct ccatgacccc cgccccgtgg gaaacctgtc catcgtgagg
 421 actaaccgcg cagagattcc catcgagtgc cgctacccca ggcagggcaa tgtgagcagc
 481 caggccatcc tgcccacctg gttgcccttc aggaccacgg tgttctcaga ggagaagctg
 541 actttctctc tgcgtctgat ggaggagaac tggaacgctg agaagaggtc ccccaccttc
 601 cacctgggag atgcagccca cctccaggca gaaatccaca ctggcagcca cgtgccactg
 661 cggttgtttg tggaccactg cgtggccaca ccgacaccag accagaatgc ctccccttat
 721 cacaccatcg tggacttcca tggctgtctt gtcgacggtc tcactgatgc ctcttctgca
 781 ttcaaagttc ctcgacccgg gccagataca ctccagttca cagtggatgt cttccacttt
 841 gctaatgact ccagaaacat gatatacatc acctgccacc tgaaggtcac cctagctgag
 901 caggacccag atgaactcaa caaggcctgt tccttcagca agccttccaa cagctggttc
 961 ccagtggaag gcccggctga catctgtcaa tgctgtaaca aaggtgactg tggcactcca
1021 agccattcca ggaggcagcc tcatgtcatg agccagtggt ccacgtctgc ttcccgtaac
1081 cgcaggcatg tgacagaaga agcagatgtc accgtggggg ccactgatct tcctggacag
1141 gagtggtgac catgaagtag agcagtgggc tttgccttct gacacctcag tggtgctgct
1201 gggcgtaggc ctggctgtgg tggtgtccct gactctgact gctgttatcc tggttctcac
1261 caggaggtgt cgcactgcct cccaccctgt gtctgcttcc gaataaaaga agaaa
```

FIG. 12A

```
                                MELSYRLFICLLLWGSTELCYPQPLWLLQGGASHPETSVQPVLV
ECQEATLMVMVSKDLFGTGKLIRAADLTLGPEACEPLVSMDTEDVVRFEVGLHECGNS
MQVTDDALVYSTFLLHDPRPVGNLSIVRTNRAEIPIECRYPRQGNVSSQAILPTWLPF
RTTVFSEEKLTFSLRLMEENWNAEKRSPTFHLGDAAHLQAEIHTGSHVPLRLFVDHCV
ATPTPDQNASPYHTIVDFHGCLVDGLTDASSAFKVPRPGPDTLQFTVDVFHFANDSRN
MIYITCHLKVTLAEQDPDELNKACSFSKPSNSWFPVEGPADICQCCNKGDCGTPSHSR
RQPHVMSQWSTSASRNRRHVTEEADVTVGATDLPGQEW
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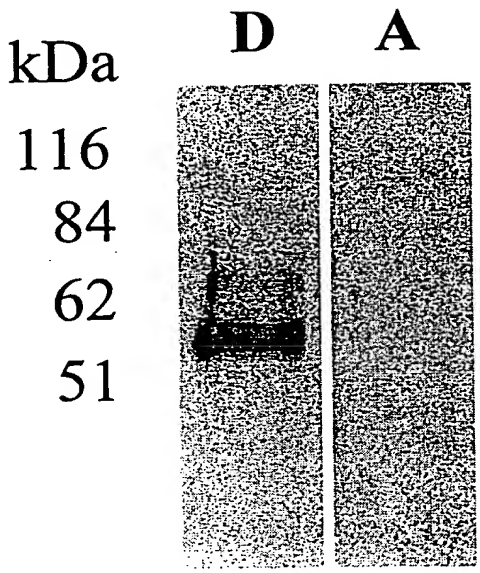
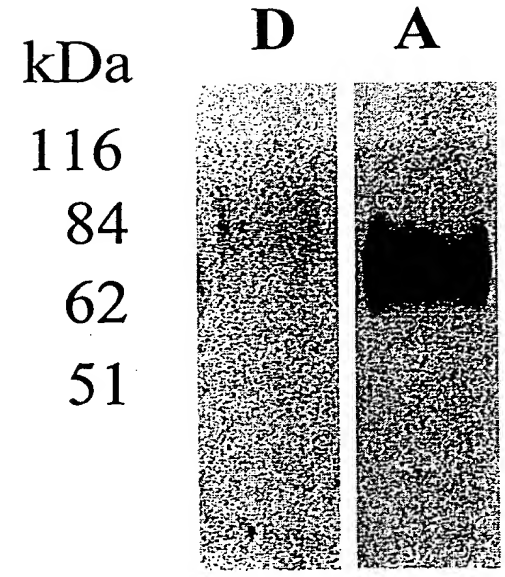
FIG. 12B



D = detergent phase

A = aqueous phase

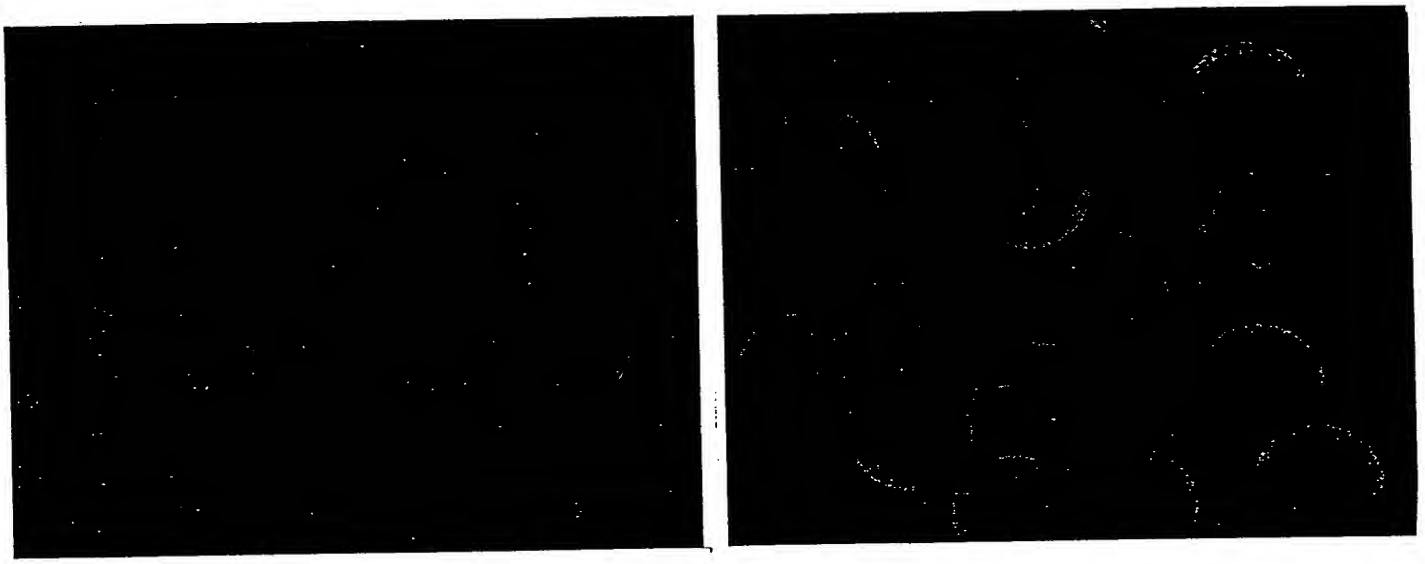
FIG. 13A



D = detergent phase

A = aqueous phase

FIG. 13B



Control: (rat IgG)

IE-10 (anti-ZP3)

FIG. 14

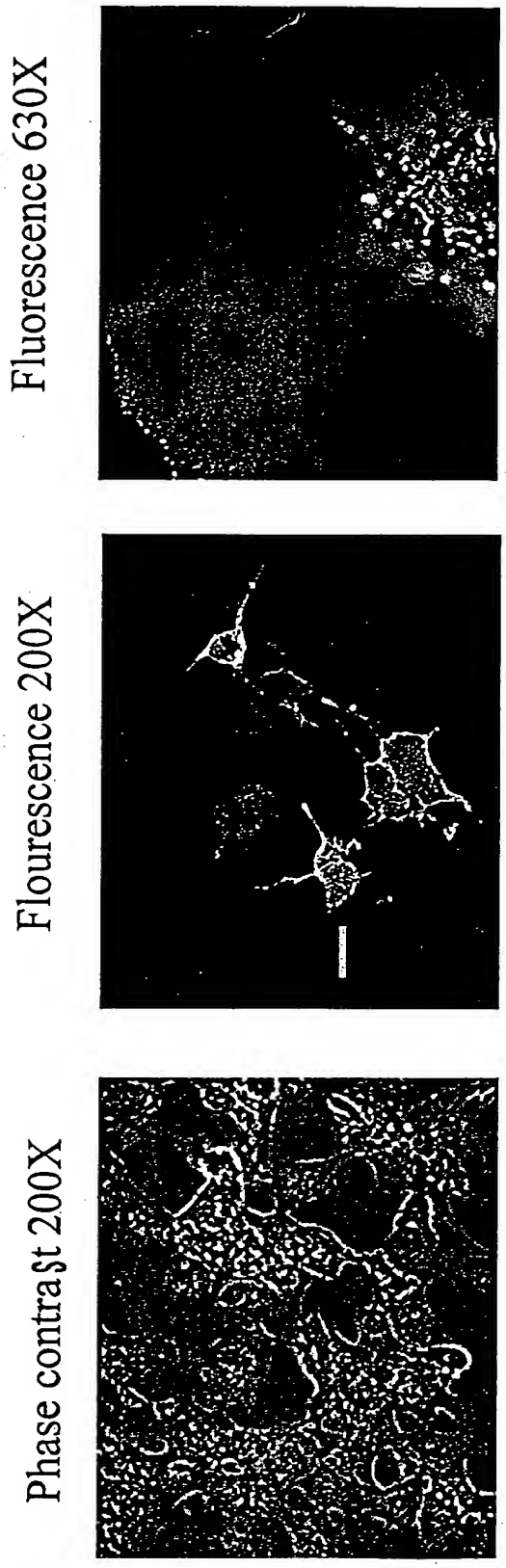


FIG. 15

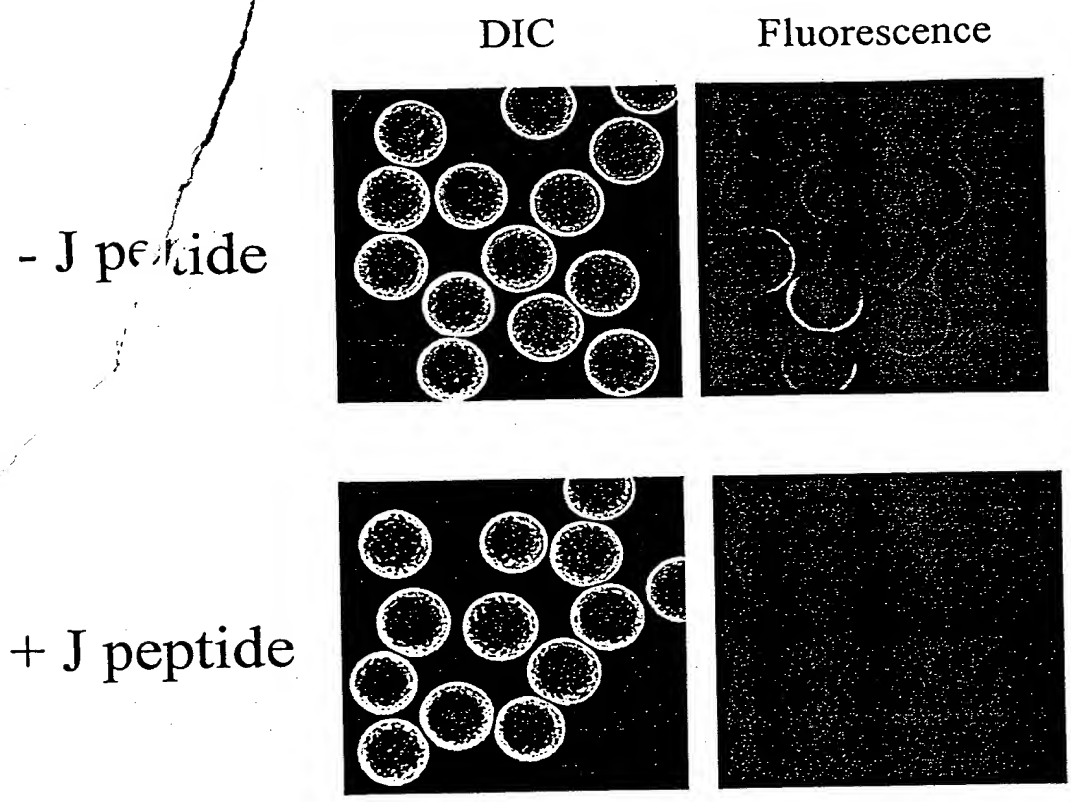


FIG. 16

US 0972028203P1

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Team: OIPEBackFileIndexing
Dossier: 09720282

Legal Date: 01-25-2001

No.	Doccode	Number of pages
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Total number of pages: 2

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